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Genetically Encoded Fluorescent Protein Biosensor for Nitric Oxide

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Genetically Encoded Fluorescent Protein Biosensor for Nitric Oxide

By

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Genetically Encoded Fluorescent Protein Biosensor for Nitric Oxide

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Nitric oxide (NO) is an important molecule in living cells for signaling, thus a specific sensor to detect its level in live cells is needed. Currently there are a few small molecule probes for the detection of NO. A common shortcoming of these probes is their unavoidable leakage from the target cells. In this project, I seek to develop a novel green fluorescent protein (GFP)-based biosensor to detect cellular NO. This GFP-based sensor is genetically encodable and can potentially avoid the possible false positive result due to the leakage. I synthesized an unnatural amino acid (unAA) and examined its incorporation into GFP to replace the chromophore-forming tyrosine 66. I also investigated the ability of this unAA to detect NO.

INDEX

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Last but not the least, I would like to thank my family: my parents for supporting me spiritually throughout writing this thesis and the assistance they provided to me in my daily life after my accident.

LIST OF ABBREVIATIONS

TLC = thin layer chromatography

NBS = N-Bromosuccinimide

DMF = dimethylformamide

$(Boc)_2O$ = di-tert-butyl dicarbonate

NMR = nuclear magnetic resonance

R. T. = room temperature

PBS = phosphate buffered saline

aq = aqueous

Me = methyl

AIBN = azobisisobutyronitrile

RBF = round button flask

EA = ethyl acetate

TFA = trifluoroacetic acid

TBAB = tetra-*n*-butylammonium bromide

DCM = dichloromethane

S. M. = starting material

HRMS = high resolution mass spectrometry

ESI-TOF = electrospray ionization mass spectrometry – time of flight

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

LB = lysogeny broth

Tet = tetracycline

Cm = chloramphenicol

Ap = ampicillin

Kan = kanamycin

unAA = unnatural amino acid

DTT = dithiothreitol

IPTG = isopropyl β-D-1-thiogalactopyranoside

Ara = arabinose

GMML = glycerol minimal media with leucine

IodoPhe = *p*-iodo-L-phenylalanine

CHAPTER 1. BACKGROUND

1.1 Synthesis and function of NO

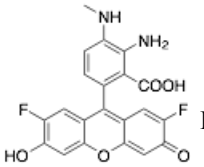
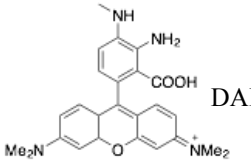
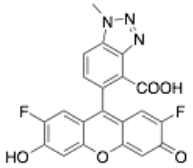
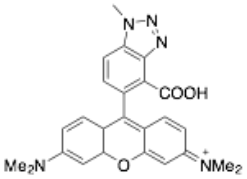
NO is a free radical with 115 pm of bond length. In the environment, NO is a byproduct of combustion of substances in the air, as in automobile engines, fossil fuel power plants, and is produced naturally during the electrical discharges of lightenings.[1] In plants, NO is mainly generated by cytosolic nitrate reductase;[2] in mammals and some bacteria, NO is generated by endothelial NO synthase (NOS), neuronal NOS and inducible NOS with separated functions. In the human body, NO is an important cellular signaling molecule involved in many physiological and pathological processes. For example, in the cardiovascular system, it regulates vasodilation and contractility of cardiac muscle;[3, 4] in the immune system, it provides anti-bacteria regulation by macrophage; it protects skin from fungi infection; it regulates inflammation and has anti-cancer activity;[5] and in the neuro system, it is a messenger via redox pathway and it also regulates learning and maintains memory.[6]

1.2 Small molecule NO biosneor

Due to the important biological functions of NO, two families of fluorescence-based bioimaging probes have been developed for real-time NO measurement (Table 1).[7, 8] However, cellular permeability is the most significant drawback of small fluorescent probes.[9] First, leakage cannot be completely avoided; second, introduction of dyes is not cell-selective. As a result, the background signal level is high and a false positive signal is observed. To overcome this drawback, GFP-based bioimaging method is considered in this project. As opposed to small-molecule dyes, GFP-based NO indicators have one major advantage: They can be targeted to tissues (by means of tissue-specific promoters), cell types, organelles, or particular cellular

domains (by fusion to protein targets). They also have no limitations posed by dye permeation or necessity for hydrolysis, where it may be inaccessible for fluorescent dyes. [10]

Table 1. Fluorescence-based small molecule probes for NO.

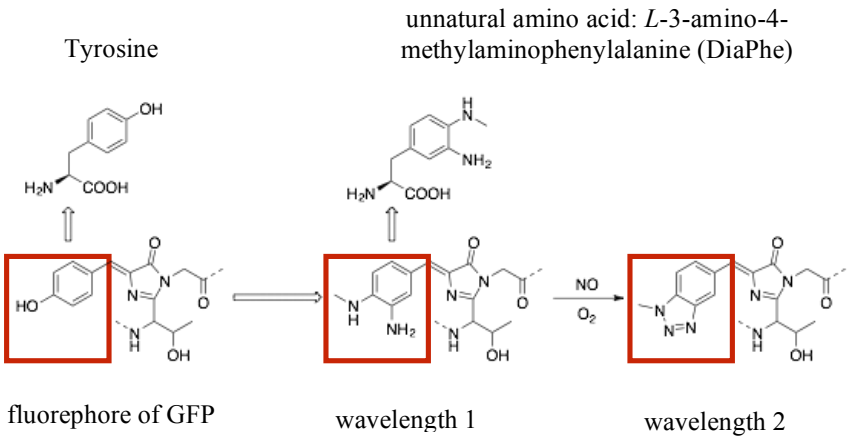
		Diaminofluoresceins (DAFs)	Diaminorhodamines (DARs)
example	dye	 DAF-FM	 DAR-4M
	product		
extinction coefficiency, absorption maximum (× 10^4 M^{-1} cm^{-1}, nm)	diamine	8.4, 487	7.8, 453
	triazole	7.3, 495	7.6, 554
wavelength of maximum fluorescence signal of triazole form (nm)		515	572
NO detection limit (nM)		3.6	7
quantum yield	diamine	0.005	0.0005
	triazole	0.81	0.42
usable pH range		above 5.8	above 4

1.3 To design a GFP-based NO sensor

Fluorescent proteins are useful probes to study biomolecules and cellular events in live cells.[11-16] In recently years, a large effort have been devoted to the use of fluorescent proteins as biosensors

for small molecule metabolites, enzyme activity, and signaling pathways in living cells.[12, 13] The traditional design and construction of fluorescent protein biosensors are limited to the common twenty amino acids. The Guo and other labs seek to use unnatural amino acids with novel physical and chemical properties to manipulate fluorescent protein structure and function in order to engineer novel properties into fluorescent proteins for biosensing. Such combination of fluorescent protein engineering with the powerful unnatural amino acid mutagenesis methodologies would provide a new frontier for the design and construction of FP biosensors.[17-22] The goal of my work is to devise and construct a fluorescent protein biosensor to detect NO in live cells.

In our design, the fluorophore of GFP is modified to contain the diamine functional group of the previously reported dyes. The GFP-based sensor may be able to detect NO based on the following mechanism:



In this project, we plan to site-specifically incorporate DiaPhe into GFP at Tyr66 position using nonsense codon suppressor tyrosyl tRNA and tyrosyl-tRNA synthetase (TyrRS). To produce the

mutated GFP, Tyr66 codon will be replaced by amber stop codon (TAG); after the induction with inducer and DiaPhe, incorporated tyrosyl tRNA/synthetase pair can recognize the codon and incorporate DiaPhe into the Tyr66 position.[23]

1.4 Unnatural amino acid mutagenesis

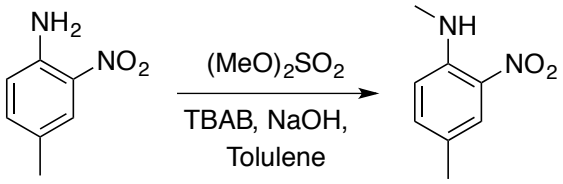
Both chemical and biosynthetic strategies have been developed to incorporate unnatural amino acids into proteins. In my research, I use the general approach that was developed by Prof. Peter Schultz for the site-specific incorporation of unnatural amino acids directly into proteins in live cells.[24]

In this system, orthogonal tRNA-aminoacyl-tRNA synthetase pairs are generated to recognize a noncoding codon (e.g., Amber nonsense codon) and not to cross-react with any of the endogenous tRNAs and aminoacyl-tRNA synthetases in the host strain. The orthogonal aminoacyl-tRNA synthetase is then modified to aminoacylate the cognate amber suppressor tRNA with only the desired unnatural amino acid. This approach allows the site-specific insertion of unnatural amino acids at a desired position with high fidelity in response to the amber (or four-base) codon and is adaptable to a large variety of amino acid side chains. By using this methodology, over 150 unnatural amino acids, including those containing spectroscopic probes, posttranslational modifications, metal chelators, photoaffinity labels, and other chemical moieties, have been selectively incorporated into proteins in bacteria, yeast, and mammalian cells with high fidelity and efficiency.[25]

CHAPTER 2 CHEMICAL EXPERIMENTS

The unnatural amino acid that I sought to synthesize is *L*-3-amino-4-methylaminophenylalanine (DiaPhe). The design of this unnatural amino acid was discussed in section 1.3 of Chapter 1. The following is the overall synthetic scheme for this unnatural amino acid.

Synthesis of compound **1**, N,4-dimethyl-2-nitroaniline



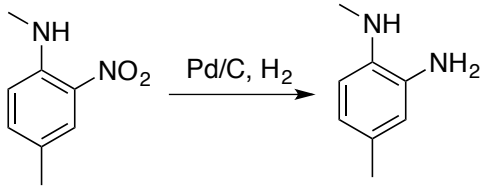
1

First, 4-methyl-2-nitroaniline (11.41g, 75mmol) was dissolved in 45 mL toluene under stirring. TBAB (2.49g, 7.5mmol) was then added into the solution followed by aqueous NaOH (9.6g, 240 mmol). The reaction was started by the dropwise addition of $(MeO)_2SO_2$. The reaction was kept at R. T. overnight and monitored by TLC. The reaction mixture was washed by water and extracted by ethyl acetate (EA). The organic layers were combined and dried by Na_2SO_4 overnight. Solvent was evaporated under reduced pressure.[26] The crude product was purified via silica gel chromatography (EA/hexane 1/20) to afford **1 (**N,4-dimethyl-2-nitroaniline) as an orange powder (11.2g, 67.44mmol, 89.9%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.01 (1H, d), 6.59 (1H, s), 6.43 (1H, d), 2.99 (3H, s), 2.33 (3H, s)

^{13}C NMR (300 MHz, $CDCl_3$) δ: 145.04, 138.02, 126.25, 124.95, 77.53, 77.22, 76.09, 29.99, 20.16

Synthesis of compound **2**, N^1,4-dimethylbenzene-1,2-diamine

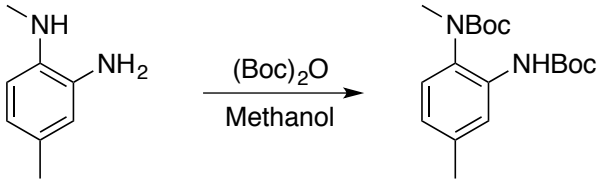


1 **2**

Compound **1** (7.2g, 43.4 mmol) was dissolved in 100 mL ethanol in round button flask. Palladium on carbon (4.6g, 4.3mmol) was added into the solution. The reaction vessel was refilled with H_2 and the reaction mixture was stirred at R. T. overnight. The reaction was monitored by TLC.[27] When the reaction was complete, the solution was filtered and used directly for the next reaction.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.38 (1H, s), 7.04 (1H, q), 6.91 (1H, s), 6.55 (1H, s)

Synthesis of compound **3**, tert-butyl (2-((tert-butoxycarbonyl)amino)-4-methylphenyl)(methyl)carbamate



2 **3**

$(Boc)_2O$ (23.7g, 108.5mmol) was melted in a water bath at 35 ℃ and then added into the solution from the previous reaction. The reaction vessel was refilled with N_2 and the mixture was

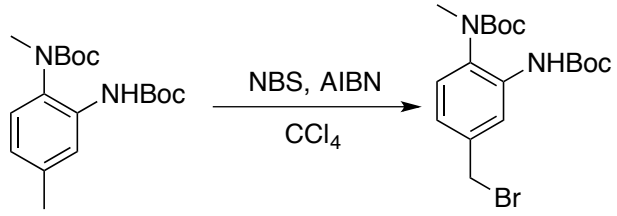
allowed to stir at R. T. overnight. Then the solvent was evaporated under reduced pressure.[28] The crude product was purified via silica gel chromatography (EA/ hexane 1/8) to afford compound **3,** tert-butyl (2-((tert-butoxycarbonyl)amino)-4-methylphenyl)(methyl)carbamate, as a white powder (13.9g, 41.34mmol, 87.5% over two steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.01(1H, s), 7.26 (3H, s), 7.16 (1H, s), 6.68 (1H, d), 4.46 (1H, s), 3.18 (3H, s), 1.53(9H, s), 1.42 (9H, s)

^{13}C NMR (300 MHz, $CDCl_3$) δ: 170.7, 155.02, 152.62, 137.45, 134.13, 126.0, 80.34, 77.30, 28.22, 21.03, 14.08

HRMS (ESI-TOF) m/z: $[M-Na]^+$: Calculated for $C_{18}H_{28}N_2O_4Na$: 359.1947; Found: 359.1954

Synthesis of compound **4**, tert-butyl (4-(bromomethyl)-2-((tert-butoxycarbonyl)amino)phenyl)(methyl)carbamate



3 **4**

Compound **3,** tert-butyl (2-((tert-butoxycarbonyl)amino)-4-methylphenyl)(methyl)carbamate (0.5g, 1.37mmol), NBS (0.243g, 1.37mmol), and AIBN (22mg, 0.1mmol) were added to the same round button flask. CCl_4 (15ml) was added and the reaction mixture was heated and kept at 78℃ under stirring overnight. The reaction progress was monitored by TLC. More NBS and AIBN were added as needed. When the reaction was complete, the solution was filtered and the solvent was evaporated under reduced pressure.[29] The crude product was purified via silica gel

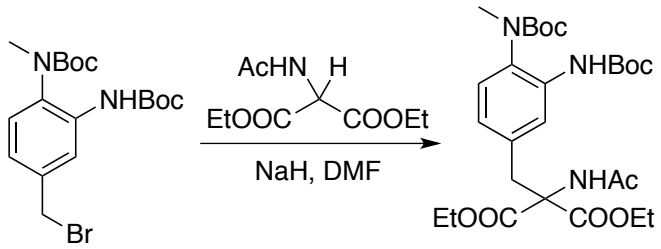
chromatography (EA/hexane 1/19) to afford compound **4,** tert-butyl (4-(bromomethyl)-2-((tert-butoxycarbonyl)amino)phenyl)(methyl)carbamate, as a light yellow powder (0.391g, 0.94mM, 69.1%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.01 (1H, s), 7.22 (1H, t), 6.67 (1H, s), 6.61 (1H, m), 4.46 (2H, s) 3.18 (3H, d), 2.18 (2H, s), 1.52 (9H, d), 1.42 (9H, s)

^{13}C NMR (300 MHz, $CDCl_3$) δ: 154.25, 152.67, 136.8, 134.22, 128.91, 82.15, 77.45, 34.52, 28.76

HRMS (EI) m/z: $[M]^+$: Calculated for $C_{18}H_{27}BrN_2O_4$: 414.1154; Found: 414.1151

Synthesis of compound **5,** diethyl 2-acetamido-2-(4-((tert-butoxycarbonyl)(methyl)amino)-3-((tert-butoxycarbonyl)amino)benzyl)malonate



4 **5**

Three round button flasks were burned by flame to remove the inner moisture. NaH (66mg, 1.64mmol) was put into one of those round button flasks, washed twice by hexane and dried under vacuum. Diethyl 2-acetamidomalonate (0.38g, 1.64mmol) was added into another round button flask and dissolved it with NaH in DMF. Two solutions were mixed under stirring with ice-water bath. Compound **4,** tert-butyl (4-(bromomethyl)-2-((tert-butoxycarbonyl)amino)phenyl)(methyl)carbamate (0.603g, 1.64mmol), was dissolved in DMF and added into the mixed solution. Then the ice-water bath was removed. The reaction was

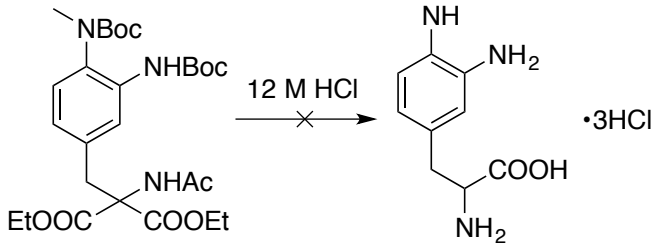
monitored by TLC every 15 minutes. When the reaction was complete, the reaction was quenched by water, the organic layer was extracted by ethyl acetate and dried over Na_2SO_4 overnight.[30] The crude product was evaporated under reduced pressure and purified via prep-TLC to afford compound **5,** diethyl 2-acetamido-2-(4-((tert-butoxycarbonyl)(methyl)amino)-3-((tert-butoxycarbonyl)amino)benzyl)malonate, as a white powder (0.8g, 2.28mM, 89.1%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.92 (1H,s), 6.88 (1H, q), 6.75 (1H, s), 6.62 (1H, s), 6.52 (1H, s), 4.25 (6H, sex), 3.60 (2H, s), 3.13 (2H, s), 1.55 (9H, t), 1.43 (9H, q), 1.29 (9H, t)

^{13}C NMR (300 MHz, $CDCl_3$) δ: 155.5, 134.26, 127.14, 78.62, 66.49, 63.86, 55.27, 38.36, 29.74

HRMS (ESI-TOF) m/z: $[M-Na]^+$: Calcd for $C_{27}H_{41}N_3O_9Na$: 574.2740; Found: 574.2726

Synthesis of compound **6,** 2-amino-3-(3-amino-4-(methylamino)phenyl)propanoic acid (failed)

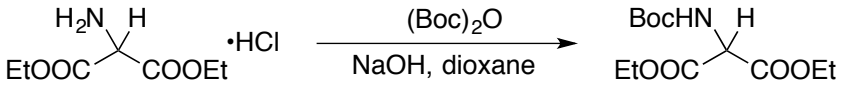


5 **6**

The compound **5,** diethyl 2-acetamido-2-(4-((tert-butoxycarbonyl)(methyl)amino)-3-((tert-butoxycarbonyl)amino)benzyl)malonate (0.603g 1.09mmol), was dissolved in water. A solution of 12 M HCl was added under N_2 protection and the resulting reaction mixture was refluxed for 12h. The reaction mixture was then extracted by DCM and evaporated under reduced pressure. The crude product was purified via prep-TLC. The NMR spectrum showed a mixture of two molecules. It is likely that the acetyl group could not be completely removed. The remaining acetyl group might have caused the observed two overlapping spectra. Similar results were

obtained when either a NaOH solution or a TFA was used to replace 12M HCl. Best result was obtained when the *tert*-butyloxycarbonyl (Boc) group was used as protecting group as it could be removed easily. (see below)

Synthesis of compound **7,** diethyl 2-((tert-butoxycarbonyl)amino)malonate



7

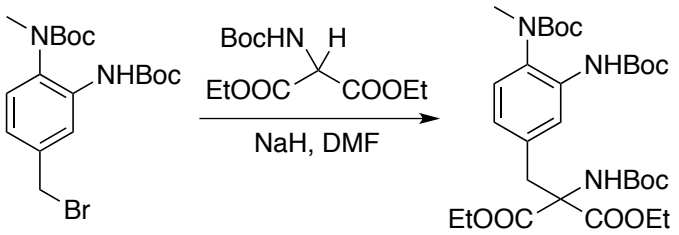
Diethyl 2-aminomalonate hydrochloride (8.4g, 40mmol) was dissolved in aqueous NaOH solution (1 M, 40ml) and dioxane. A solution of $(Boc)_2O$ (9.6g, 44mmol) in dioxane (50ml in total) was added into the solution of diethyl 2-((tert-butoxycarbonyl)amino)malonate under ice-water bath. After 30 min, the ice-water bath was removed and the reaction was kept at R. T. overnight. The organic layer was extracted by EtoAc and evaporated under reduced pressure.[31] The crude product was purified via silica gel chromatography to afford compound **7,** diethyl 2-((tert-butoxycarbonyl)amino)malonate, as a colorless oil (6.0g, 21.8mM, 58.4%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.55 (1H, d), 4.86 (1H, d), 4.21 (4H, m), 1.38 (9H, s), 1.22 (6H, t)

^{13}C NMR (300 MHz, $CDCl_3$) δ: 166.84, 154.66, 80.27, 62.21, 57.42, 28.03, 13.82

HRMS (ESI-TOF) m/z: $[M-Na]^+$: Calculated for $C_{12}H_{21}NO_6Na$: 298.1267; Found: 298.1279

Synthesis of compound **8,** diethyl 2-(4-((tert-butoxycarbonyl)(methyl)amino)-3-((tert-butoxycarbonyl)amino)benzyl)-2-((tert-butoxycarbonyl)amino)malonate



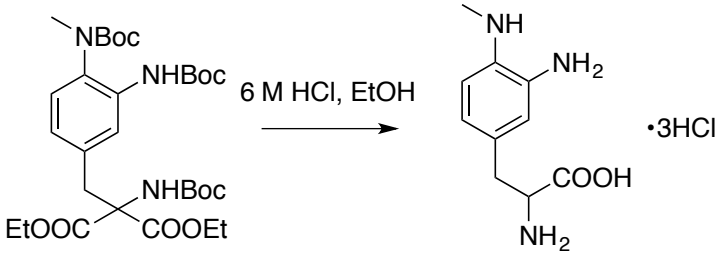
4 **8**

This reaction is similar to the synthesis of compound **5**. Three round button flasks were heated by flame to remove the inner moisture. NaH (1.344g, 33.6mmol) was added into one of those round button flasks, washed twice by hexane and dried in vacuum. Diethyl 2-((tert-butoxycarbonyl)amino)malonate (9.24g, 33.6mmol) was added in another round button flask and dissolved in a DMF solution of NaH. These two solutions were mixed under stirring and ice-water bath. Compound **4,** tert-butyl (4-(bromomethyl)-2-((tert-butoxycarbonyl)amino)phenyl)(methyl)carbamate (11.6g, 28mmol), was dissolved in DMF and added into the reaction mixture. After 15 minutes, the ice-water bath was removed. The reaction was monitored by TLC every 15 min. When the reaction was complete, the reaction was quenched by water. The organic layer was extracted by EtOAc and dried over Na_2SO_4 overnight. Then the organic layer was evaporated under reduced pressure. The crude product was purified via prep-TLC to afford compound **8,** diethyl 2-(4-((tert-butoxycarbonyl)(methyl)amino)-3-((tert-butoxycarbonyl)amino)benzyl)-2-((tert-butoxycarbonyl)amino)malonate, as a yellow powder(7.8g, 12.8 mmol, 45.71%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.85 (1H, s), 6.84 (1H, d), 6.71 (1H, s), 6.57 (1H, s), 4.15 (4H, sex), 3.85 (2H, s), 3.07 (3H, s), 1.39 (27H, quin), 1.34 (6H, t)

HRMS (ESI-TOF) m/z: $[M-Na]^+$: Calculated for $C_{30}H_{47}N_3O_{10}Na$: 632.3159; Found: 632.3154

Synthesis of compound **6,** 2-amino-3-(3-amino-4-(methylamino)phenyl)propanoic acid



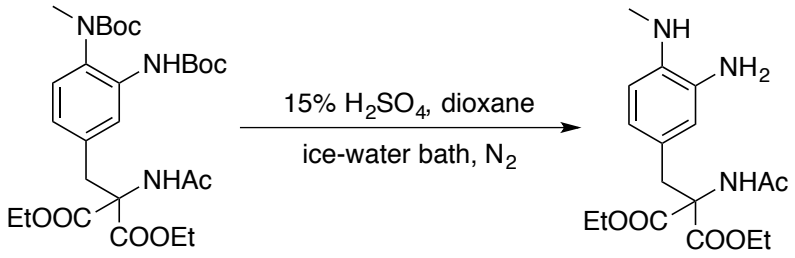
8 **6**

This reaction is similar to the synthesis of compound **6,** 2-amino-3-(3-amino-4-(methylamino)phenyl)propanoic acid. Compound **8**, diethyl 2-(4-((tert-butoxycarbonyl)(methyl)amino)-3-((tert-butoxycarbonyl)amino)benzyl)-2-((tert-butoxycarbonyl)amino)malonate (916mg, 1.5mmol), was dissolved in 10 mL 6M HCl and 10 mL EtOH. The reaction mixture was refluxed under N_2 protection overnight. Then the solution was directly evaporated under reduced pressure to afford compound **6,** 2-amino-3-(3-amino-4-(methylamino)phenyl)propanoic acid, as a yellow powder. (283mg, 1.35 mmol, 90%)

^{1}H NMR (400 MHz, D_2O) δ: 7.27 (1H, d), 7.01 (2H, sex), 3.26 (2H, sep), 3.04 (3H, s)

^{13}C NMR (300 MHz, $CDCl_3$) δ: 171.24, 134.13, 123.32, 121.71, 121.08, 53.84, 35.06, 34.95, 13.11

Synthesis of Compound **9,** diethyl 2-acetamido-2-(3-amino-4-(methylamino)benzyl)malonate:



5 **9**

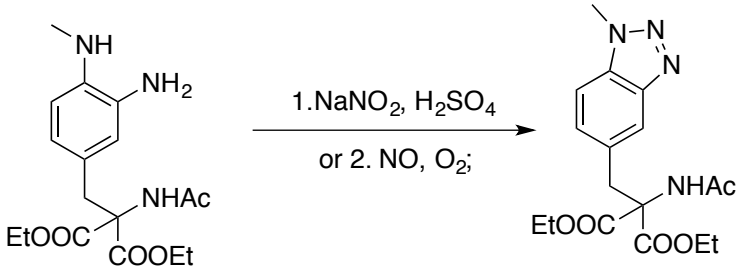
A round button flask was filled by N_2 and put on the ice-water bath. Compound **5** (52mg, 0.094mmol) was dissolved in dioxane (1 mL) and transferred into the round button flask. A solution of 15% H_2SO_4 (1 mL) was added under stirring, N_2 and ice-water bath. After 30 minutes, the ice-water bath was removed and the reaction was kept at R. T. for 1h. The reaction was then neutralized by the addition of 15 mL $NaHCO_3$. The solution was extracted by EtOAc and the organic layer was dried over Na_2SO_4 overnight under N_2 protection. The crude product was purified by prep-TLC to afford compound **9,** diethyl 2-acetamido-2-(3-amino-4-(methylamino)benzyl)malonate, as a yellow powder.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.27 (1H, s), 6.58 (1H, s), 6.44 (1H, s), 4.27 (4H, m), 3.54 (1H, s), 2.93 (2H, s), 2.18 (3H, s), 1.30 (6H, quin)

HRMS (ESI-TOF) m/z: $[M-Na]^+$: Calculated for $C_{17}H_{25}N_3O_5Na$: 374.1692; Found: 374.1703

To prove the di-amine group was reactive toward NO, I tried some analytical experiments, but none was successful (see chapter 2 for details). I had to go back to organic experiments to proof its reactivity. I designed two parallel reactions:

1. Synthesis of compound **10,** diethyl 2-acetamido-2-((1-methyl-1H-benzo[d][1,2,3]triazol-5-yl)methyl)malonate in first pathway:



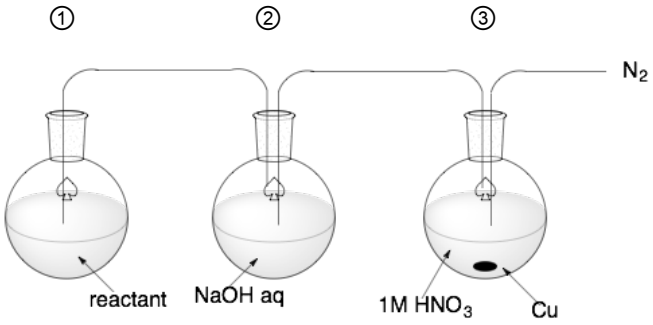
9 **10**

Compound **7** (52mg, 0.15mmol) was dissolved in 1 mL dioxane under ice-water bath. A solution of 1 mL 15% H_2SO_4 was added into the solution. After 30 minutes, the reaction was checked by TLC and it was complete. $NaNO_2$ (12mg) was dissolved in 0.5 mL water and added into the solution under N_2, stirring and ice-water bath for 5 min. Then the ice-water bath was removed and the reaction was run for an additional 1h. The solution was neutralized by saturated $NaHCO_3$, extracted by EtOAc and dried over Na_2SO_4 for 2h.[32] The solvent was evaporated under reduced pressure. The crude product was purified by prep-TLC to afford compound **10,** diethyl 2-acetamido-2-((1-methyl-1H-benzo[d][1,2,3]triazol-5-yl)methyl)malonate, as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.71 (1H, s), 7.44 (1H, d), 7.27 (1H, s), 7.20 (1H, d), 6.54 (1H, s), 4.30 (8H, q), 3.84 (2H, s), 2.06 (3H, s), 1.32 (6H, q)

HRMS (ESI-TOF) m/z: $[M-Na]^+$: Calculated for $C_{17}H_{22}N_4O_5Na$: 385.1488; Found: 385.1471

2. The NO generation device in second pathway:



All the water was degassed in this reaction by a vacuum filter device for 30min. A solution of NaOH (50 mL, 2M) and 1M HNO_3 (100ml, 1M) were made using degassed water. Aqueous NaOH solution, Cu (1.28g, 20mmol), and fresh compound **9** were added to three individual

flasks. The device was vacuumed and refilled by N_2. The reaction was started by the addition of 1M HNO_3. After 1 h, a Griess assay was conducted to check NO concentration (Table 2).

Table 2. Generation of NO.

layout:	standard conc. (mM)	standard conc. (mM)	solution dilution	solution dilution
A	100	100	10	10
B	50	50	100	100
C	25	25	1000	1000
D	12.5	12.5		
E	6.25	6.25		
F	3.13	3.13		
G	1.58	1.58		
H	0	0		
result:	standard conc. (mM)	standard conc. (mM)	solution dilution	solution dilution
A	0.84	0.838	overflow	overflow
B	0.461	0.436	1.793	1.806
C	0.259	0.253	0.258	0.261
D	0.157	0.163		
E	0.101	0.125		
F	0.073	0.072		
G	0.06	0.06		
H	0.045	0.045		

The concentration of NO in 1000-fold dilution solution was 26.17 mM. The concentration was high enough for the next step. After 1h, the solution turned acidic. To avoid affecting the

reaction by the change of pH, compound **9** in round button flask 3 should be dissolved in 1 : 1 MeCN and PBS buffer.

To do this, compound **9** (60mg 0.17mmol) was dissolved in 1 : 1 MeCN and PBS (20ml) under stirring. NO was babbled into the solution for 1 h. The solution was then evaporated under reduced pressure and the reaction product was purified via prep-TLC to afford compound **10**.

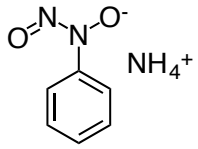
^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.71 (1H, s), 7.43 (1H, d), 7.19 (1H, d), 6.55 (1H, s), 4.29 (7H, s), 3.84 (2H, s), 2.06 (3H, s), 1.31 (6H, q)

HRMS (ESI-TOF) m/z: $[M-Na]^+$: Calculated for $C_{17}H_{22}N_4O_5Na$: 385.1488; Found: 385.1482

Two above products were identical, which proved the reactivity between NO and the di-amine group of my amino acid.

CHAPTER 3 ANALYTICAL EXPERIMENT

In this section, I attempted to use HPLC to detect the new signal of the compound after the diamine group of DiaPhe reacted with NO to generate a triazole group. First, it was needed to find a proper NO donor which could stably release NO. Then it was examined if the released NO could react with DiaPhe. The last step would be to detect the new signal from the new compound through HPLC.



cupferron

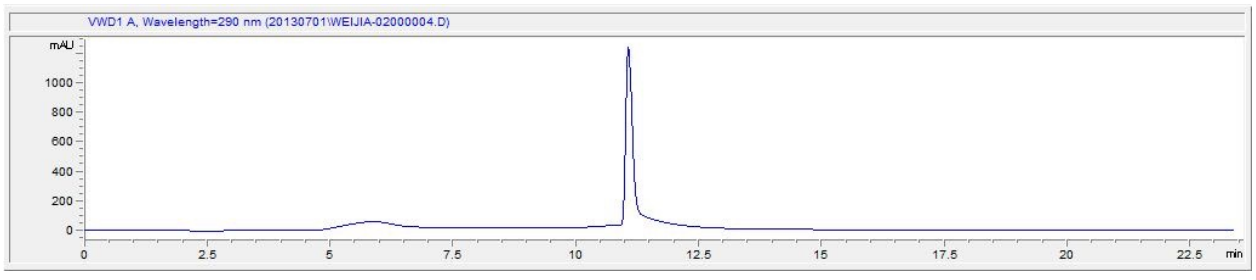
I examined cupferron[33] as a NO donor first. The releasing condition of NO of cupferron was under UV exposure.

The following HPLC protocol 1 was setup:

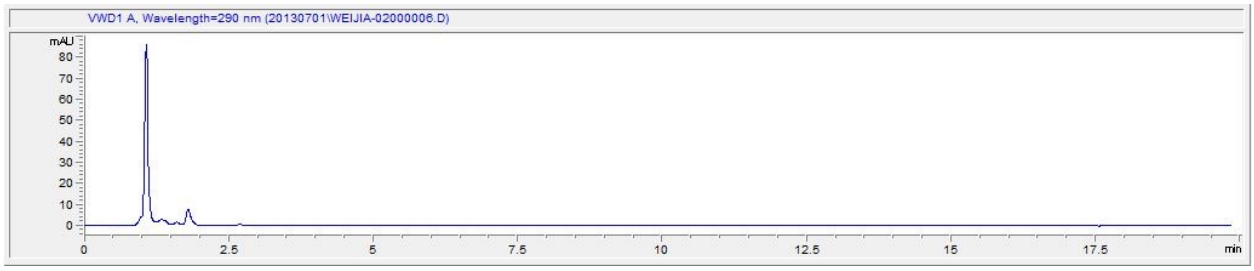
0	phosphate buffer	100%
10min	phosphate buffer methanol	75% 25%

The following were results:

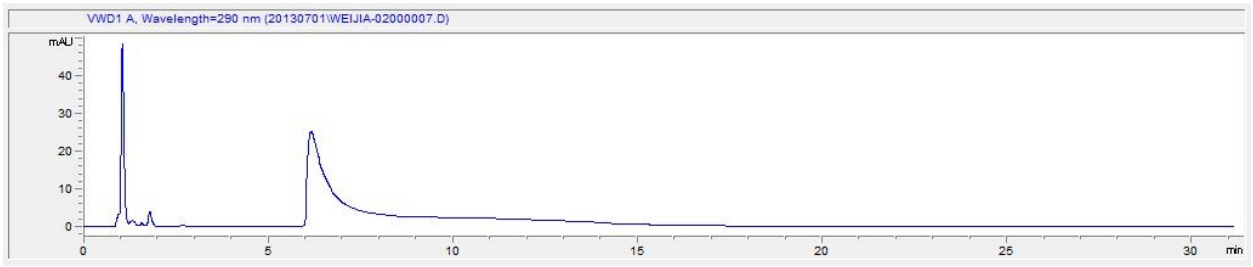
The spectrum of 1mM cupferron in 0.1 M PBS buffer (pH 7.4) was obtained:



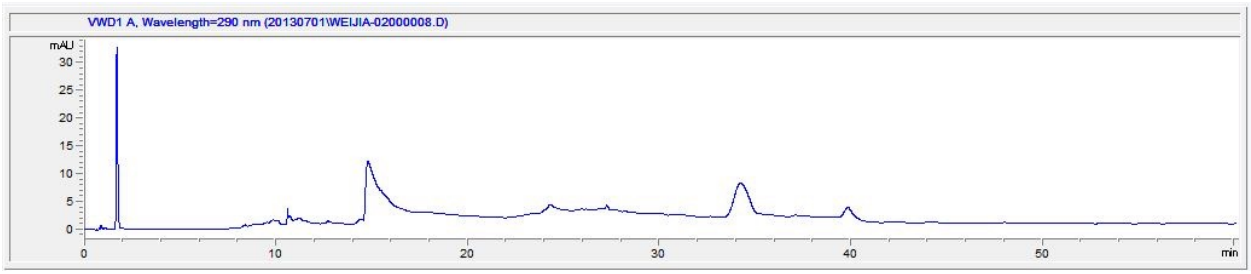
The spectrum of 1mM DiaPhe in 0.1M PBS (pH 7.4) was obtained:



The spectrum of a mixture of 0.5 mM DiaPhe and 0.5mM cupferron after 1 h UV exposure in 0.1 PBS (pH 7.4) was obtained:



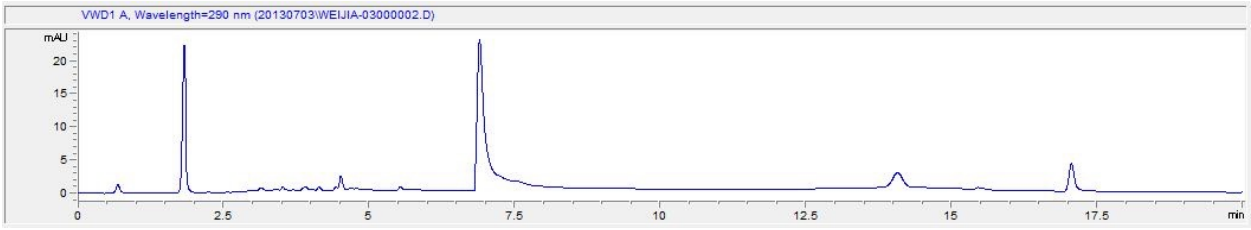
The spectrum of a mixture of 0.5mM DiaPhe and 0.5mM cupferron after 4 h UV exposure in 0.1 phosphate buffer was obtained:



Next, I set up HPLC protocol 2:

0	water phosphate buffer (pH 7.4)	10% 90%
5 to 10min	water phosphate buffer (pH 7.4) MeCN	70% 10% 20%
15min	water phosphate buffer (pH 7.4) MeCN	50% 10% 40%

By using this new protocol, I attempted to investigate the HPLC spectrum of the mixture of 0.5mM DiaPhe and 0.5mM cupferron after 4 h UV exposure in 0.1 phosphate buffer:



Since there was no identifiable signal in the HPLC spectrum, I tried Sodium Nitroprusside ($Na_2[Fe(CN)_5NO]\cdot 2H_2O$, SNP) as a NO donor.[34] Its releasing condition of NO is also under UV exposure. 10 mM SNP in PBS was made. After 1 h UV exposure, NO concentration was measured by Griess assay:

layout:	standard conc. (mM)	standard conc. (mM)	standard conc. (mM)	solution conc. (mM)
A	100	100	100	1
B	50	50	50	1
C	25	25	25	

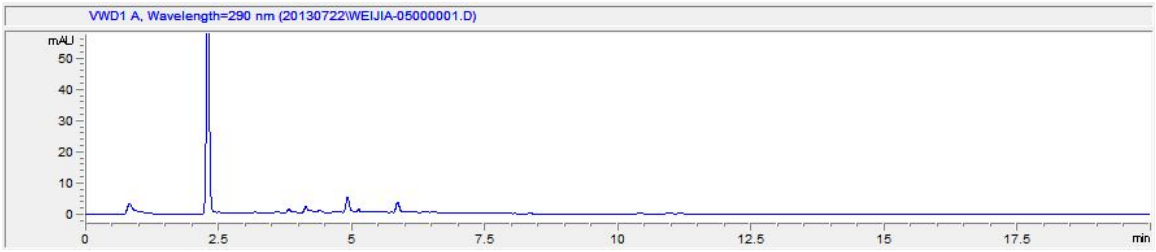
D	12.5	12.5	12.5	
E	6.25	6.25	6.25	
F	3.13	3.13	3.13	
G	1.58	1.58	1.58	
H result:	0 standard conc. (mM)	0 standard conc. (mM)	0 standard conc. (mM)	solution dilution
A	0.948	0.957	0.937	0.061
B	0.516	0.509	0.519	0.061
C	0.29	0.293	0.294	
D	0.169	0.161	0.169	
E	0.105	0.105	0.106	
F	0.073	0.073	0.071	
G	0.056	0.058	0.057	
H	0.04	0.039	0.039	

The concentration of NO was 2.035mM, which was low.

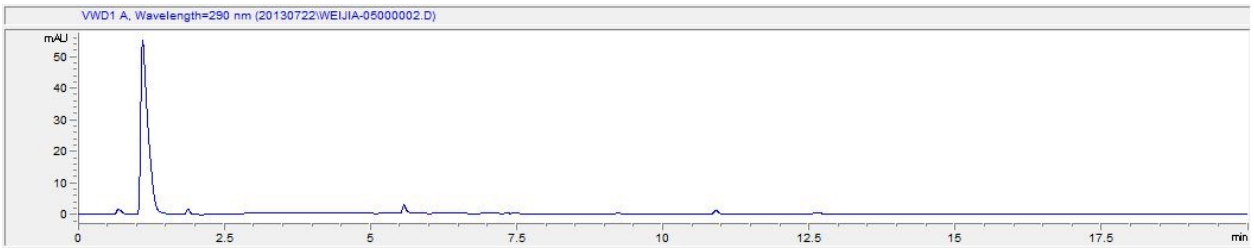
To better identify the signal in HPLC, I set up HPLC protocol 3:

0	water:phosphate buffer (pH 7.4):MeCN	90%:10%:0%
10min	water:phosphate buffer (pH 7.4):MeCN	50%:10%:40%

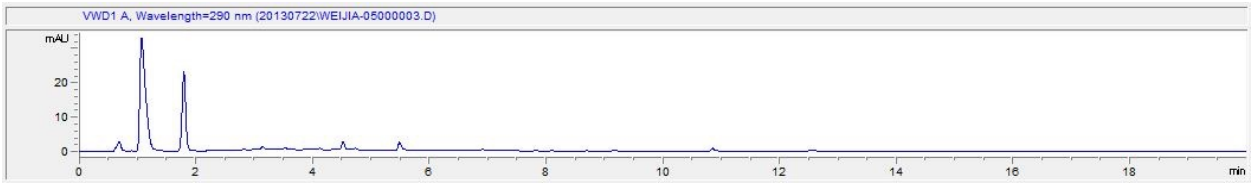
First it was tried to run HPLC to analyze 1mM DiaPhe in PBS (pH 7.4):



Then it was tried to investigate the HPLC spectrum of 1 mM SNP in PBS (pH 7.4) after 1 h UV exposure:



Finally, it was investigated HPLC spectrum of 1 : 1 mixture of 1mM DiaPhe and 1mM SNP in PBS after 1h UV exposure was occurred:



Based on previous report, the existence of cysteine can accelerate the release of NO by SNP.[35] To verify that, Griess assay was performed with 10 mM SNP only and 10mM SNP with 25mM CYS after 1 – 6 h of UV exposure:

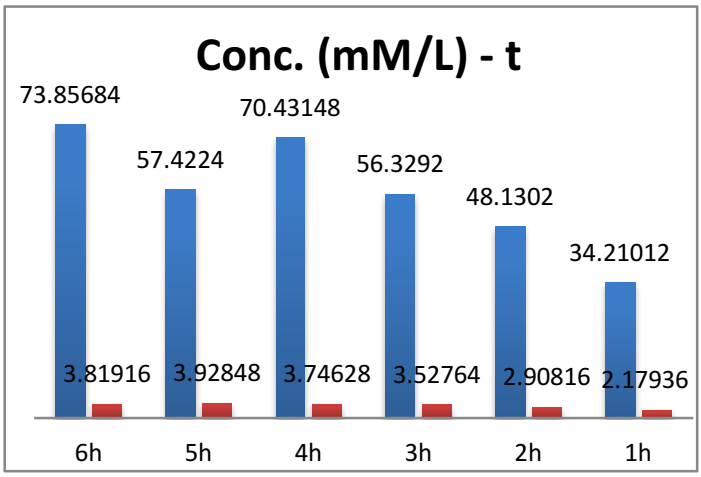
UV exposure time	6h	5h	4h	3h	2h	1h
10mM SNP	SPL2	SPL4	SPL6	SPL8	SPL10	SPL12
10mM SNP 25mM CYS	SPL1	SPL3	SPL5	SPL7	SPL9	SPL11

The result was shown below:

	1	2	3	4	5	6	7	8
A	STD1	STD1	STD1	SPL1	SPL3	SPL6	SPL9	SPL11
	100	100	100					
B	STD2	STD2	STD2	SPL1	SPL4	SPL6	SPL9	SPL12
	50	50	50					
C	STD3	STD3	STD3	SPL1	SPL4	SPL7	SPL9	SPL12
	25	25	25					
D	STD4	STD4	STD4	SPL2	SPL4	SPL7	SPL10	SPL12
	12.5	12.5	12.5					
E	STD5	STD5	STD5	SPL2	SPL5	SPL7	SPL10	
	6.25	6.25	6.25					
F	STD6	STD6	STD6	SPL2	SPL5	SPL8	SPL10	
	3.13	3.13	3.13					
G	STD7	STD7	STD7	SPL3	SPL5	SPL8	SPL11	
	1.56	1.56	1.56					
H	STD8	STD8	STD8	SPL3	SPL6	SPL8	SPL11	
	0	0	0					

	1	2	3	4	5	6	7	8
A	0.953	0.954	0.956	0.716	0.718	0.735	0.501	0.366
B	0.513	0.517	0.515	0.081	0.084	0.082	0.488	0.064
C	0.28	0.285	0.287	0.573	0.58	0.565	0.474	0.064
D	0.166	0.166	0.166	0.082	0.084	0.084	0.074	0.074
E	0.104	0.104	0.104	0.684	0.7	0.691	0.073	
F	0.073	0.072	0.073	0.083	0.083	0.079	0.075	
G	0.057	0.058	0.057	0.556	0.568	0.564	0.352	
H	0.042	0.041	0.04	0.079	0.081	0.079	0.363	

The result proved that cysteien could accelerate the release of NO from SNP.



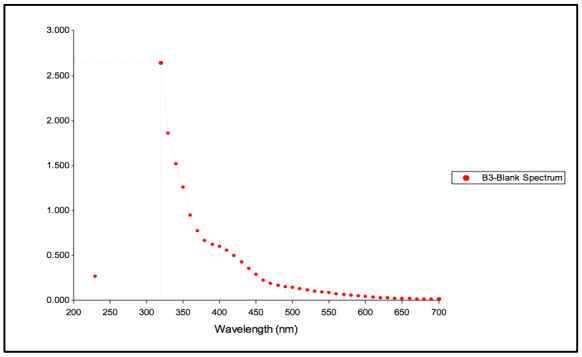
I also compared to the NO release rate of SNP with EDTA. Griess assay was done for 10 mM SNP and 25mM EDTA after 1 h of UV exposure was performed:

layout	4	5	6	7
A		STD1	STD1	STD1
		100	100	100
B		STD2	STD2	STD2
		50	50	50
C	SPL1	STD3	STD3	STD3
		25	25	25
D	SPL1	STD4	STD4	STD4
		12.5	12.5	12.5
E		STD5	STD5	STD5
		6.25	6.25	6.25
F		STD6	STD6	STD6
		3.13	3.13	3.13
G		STD7	STD7	STD7
		1.56	1.56	1.56
H		STD8	STD8	STD8
		0	0	0

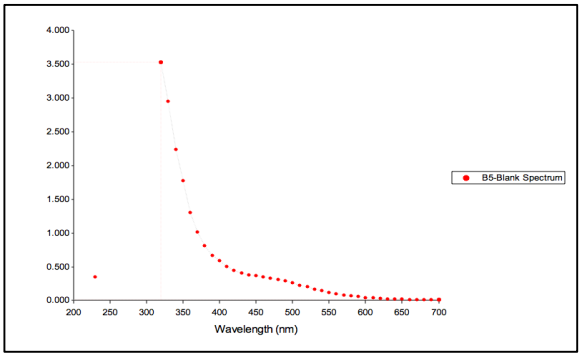
result	4	5	6	7
A		0.952	0.928	0.927
B		0.492	0.502	0.498
C	0.379	0.28	0.281	0.267
D	0.374	0.176	0.163	0.162
E		0.1	0.102	0.1
F		0.071	0.072	0.071
G		0.055	0.056	0.056
H		0.04	0.04	0.04

The concentration of NO is 41.5 μM/L, which was lower than that from SNP with cysteine.

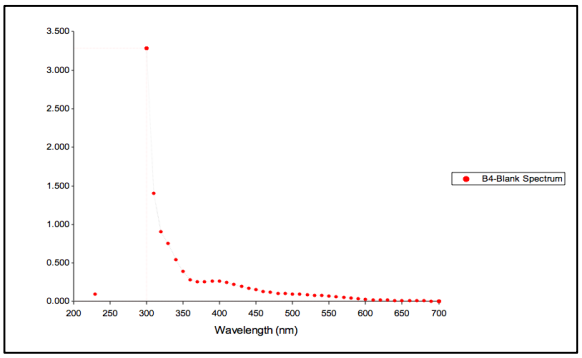
Then I tried to examine the reaction between compound **6** and NO by monitoring difference in UV spectrum. To this end, fresh compound **6** was prepared and mixed with SNP and cysteine under UV exposure. Compound **8** was used as a reference:



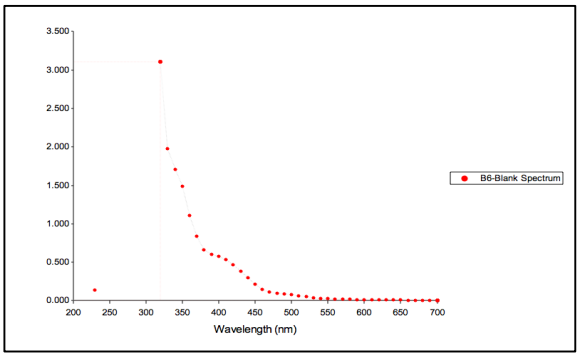
compound 6 + SNP + CYS



compound 6 + $NaNO_2$



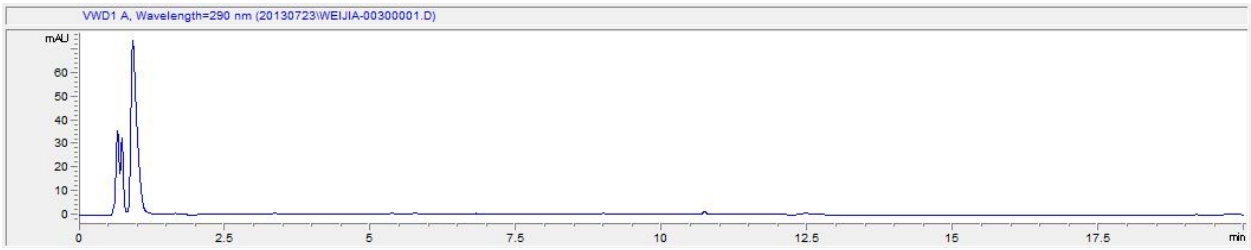
SNP



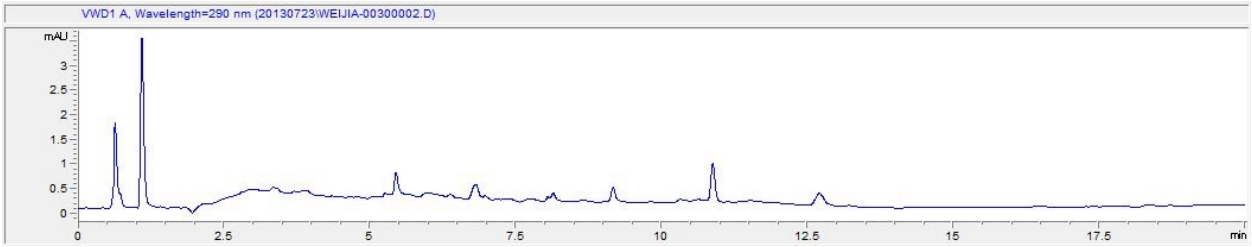
compound 6

No difference in UV spectrum was observed. Then I checked HPLC to see if there was any reaction between compound **6** and NO (protocol 3).

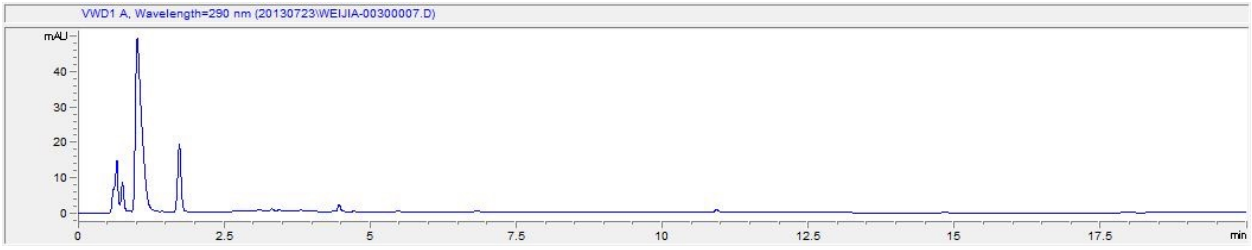
First, I examined the HPLC spectrum of 1 mM SNP with 25 mM cysteine after 1.5 h of UV exposure:



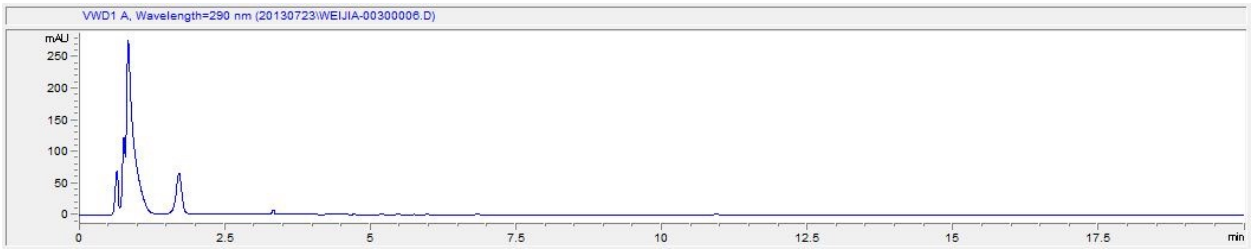
Then I attempted to observe the HPLC spectrum of pure 25mM cysteine:



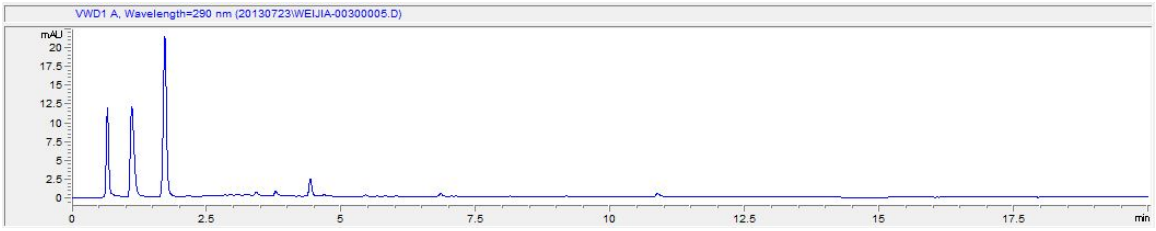
I tried to observe the HPLC spectrum of 0.5mM compound **6** with 0.5mM SNP and 1.25mM cysteine after 6 h of UV exposure:



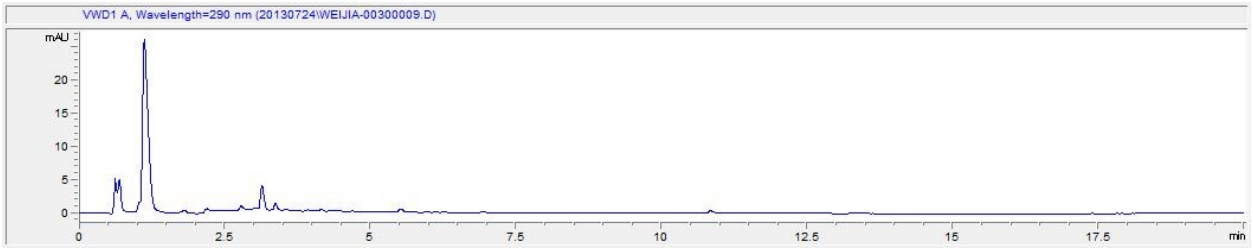
I tried to analyze the HPLC spectrum of 0.5mM compound **6** with 5mM SNP and 25mM cysteineafter 6 h of UV exposure:



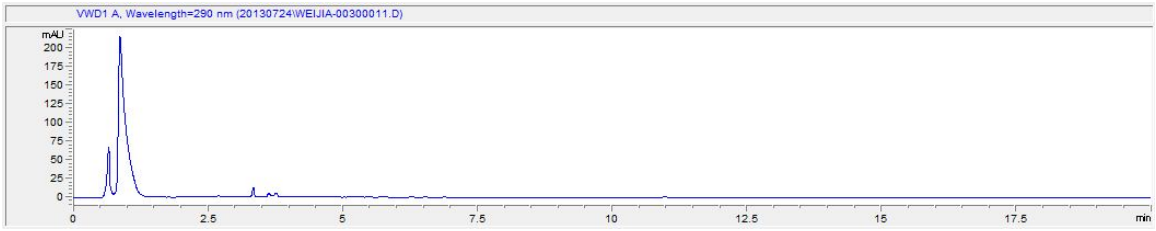
I attempted to investigate the HPLC spectrum of 0.5mM compound **6** with 5mM $NaNO_2$ and HCl after 3 h of reaction:



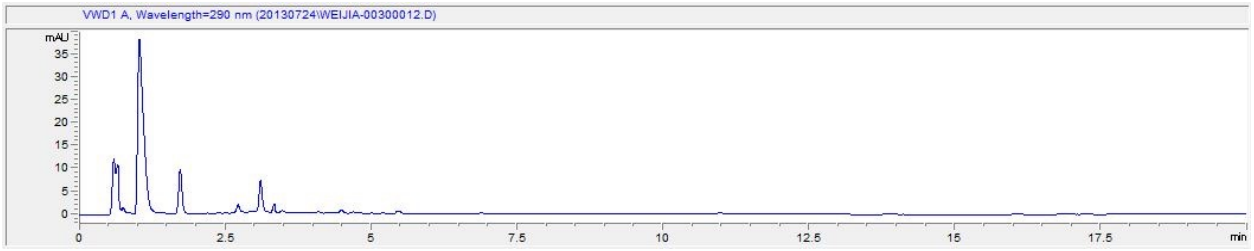
I tried to analyze the HPLC spectrum of 0.5mM compound **6** after 1d storage:



I attempted to investigate the HPLC spectrum of 0.5mM compound **6** with 5mM SNP and 12.5mM cysteine after 1 d of UV exposure:



Finally, I tried to analyze the HPLC spectrum of 0.5mM compound **6** with 0.5mM SNP and 1.25mM cysteine after 1 d of UV exposure:



Conclusion: there was no convincing evidence to prove that compound **6** could react with NO. I would have to prove this reaction by conducting chemical synthesis (see details in Chapter 1).

CHAPTER 4 BIOLOGICAL EXPERIMENTS

Since the reactivity of DiaPhe (compound **8**) with NO had been verified by chemical synthesis, the next step would be the introduction of DiaPhe into GFP and to investigate its reaction with NO in live cells.

1. Introduction of DiaPhe into proteins by using the JM3 library:

   I attempted to see if any aminoacyl-tRNA synthetase mutant from the JM3 library could recognize DiaPhe.

   a. Cell growth media and library preparation:

LB broth	50 ml
Kan_{50}	50 μl
Cm_{34}	74 μl
Tet_5	125 μl
JM3 lib	50 μl

   Cells containing the JM3 library were incubated at 37℃ overnight. On the next day, no colony was observed. Then I realized there should be no Cm in the growth media. The incubation was repeated with the correct antibiotics:

LB broth	50 ml
JM3 lib	50 μl
Kan_{50}	50 μl
Tet_5	125 μl

   On the next day, the OD_{600} value of the media was determined as 4.1.

b. The preparation of the plate with GMML media:

Plate	1	2
Agar (ml)	38	38
Tet_5 (µl)	125	125
Cm_{34} (µl)	74	74
Kan_{50} (µl)	50	50
P-I-Phe (mg)	15	0
5 X M9 salt (ml)	10	10
50% glycerol (ml)	1	1
25% NaCl (ml)	1	1
0.3M leucine (µl)	50	50
1M $MgSO_4$ (µl)	50	50
0.5M $CaCl_2$ (µl)	10	10

Cells containing the JM3 library was collected, washed with 1 ml water, and centrifuged. The supernatant was discarded and cells was resuspended in 1 mL 1X M9 salt solution. Cell suspension (55 µl) and 145 µl of water were transferred on the plate. Cells were evenly spread and incubated at 37℃ overnight. However, no colonies were observed on the next day.

I repeated the positive selection. On the next day, there were 9 colonies with fluorescence. The plate was stored for further experiment.

c. Examine the JM3 library with a control unnatural amino acid:

Plate	1	2	3
Agar (ml)	50	50	50
Tet_5 (µl)	125	125	125
Kan_{50} (µl)	50	50	50
Cm_{34} (µl)	74	74	74
DiaPhe (mg)	0	16	0
P-I-Phe (mg)	0	0	15

I performed the positive selection and observed the following cell growth:

plate	colonies	Colonies with fluorescence
1	65	27
2	56	22
3	49	19

These colonies were kept for further experiments.

2. Introduction of DiaPhe into proteins by using the JW12N library:

I attempted to see if any aminoacyl-tRNA synthetase mutants from the JW12N library could recognize DiaPhe.

a. The preparation of the JW12N library:

LB broth	50 ml
Kan_{50}	50 µl
Cm_{34}	74 µl
Tet_5	125 µl
JW12N lib	50 µl

The cells was incubated under 37℃ overnight. Similar to above, on the next day, no colony was observed because there should be no Cm in the media. The cell growth was then repeated:

LB broth	50 ml
JW12N lib	50 µl
Kan_{50}	50 µl
Tet_5	125 µl

On the next day, I determined the OD_{600} value of the media as 2.9.

b. The preparation of 150 ml GMML media:

5X M9 salt	30 ml
50% glycerol	3 ml
0.3M L-leucine	150 µl
1M $MgSO_4$	150 µl

25% NaCl	3 ml
Agar	114 ml

The preparation of plates:

plate	1	2	3
GMML (ml)	50	50	50
Tet_5 (µl)	125	125	125
Kan_{50} (µl)	50	50	50
Cm_{34} (µl)	74	74	74
DiaPhe (mg)	0	16	0
P-I-Phe (mg)	0	0	15

Cells containing the JW12N library was collected, washed by water, and resuspended in 1 ml of 1X M9 salt solution. Cells (69 µl with 131 µl autoclaved nano water) were transferred to GMML plate and incubated at 37 ℃ overnight. On the next day, however, no colonies were observed. The incubation was repeated on GMML plate. On the next day, there were 48 colonies with fluorescence, 23 colonies with strong fluorescence. The plate was kept for further investigate.

c. Selection using a different media:

LB agar	50 ml
Kan_{50}	50 µl
Tet_5	125 µl
Cm_{34}	74 µl
DiaPhe	15 mg
DTT	4 mg

Cells containing the JW12N library were transferred on the plate and incubated at 37℃ overnight. On the next day, there were 48 colonies with fluorescence, 20 colonies with strong fluorescence. Kept the plate for further experiment.

3. Plate preparation for cell replication:

In order to examine if the above clones can specifically recognize DiaPhe, they were picked and their growth was examined with or without DiaPhe.

a. The preparation of the plates with common media:

plate	1	2
LB agar (ml)	50	50
Kan_{50} (μl)	50	50
Cm_{34} (μl)	74	74
Tet_5 (μl)	125	125
DiaPhe (mg)	15	0
DTT (mg)	4	4

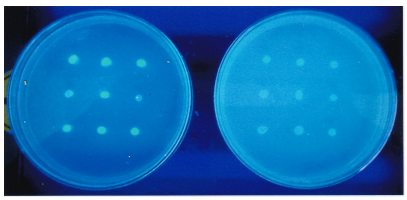
b. The preparation of the plates with GMML media:

Plate	1	2
5X M9 salt (ml)	10	10
Agar (ml)	~38	~38
50% glycerol (ml)	1	1
25% NaCl (ml)	1	1
0.1% leucine (μl)	150	150
1M $MgSO_4$ (μl)	50	50
0.5M $CaCl_2$ (μl)	10	10
Kan_{50} (μl)	50	50
Tet_5 (μl)	125	125
Cm_{34} (μl)	74	74
DiaPhe (mg)	15	0
DTT (mg)	4	4

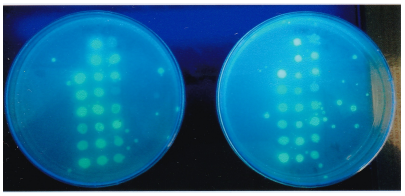
c. Colonies with fluorescence from previous experiments were replicated to plates:

Colonies from previous experiments	Replicated to
9 colonies of JM3 on GMML plate	GMML plate
22 colonies of JM3 on common plate	Common plate
48 colonies of JW12N on GMML plate	GMML plate
48 colonies of JW12N on common plate	Common plate

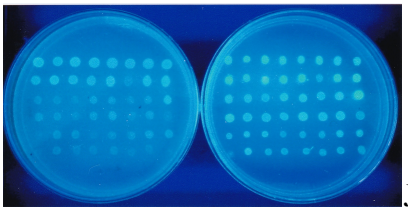
d. The result of replication:



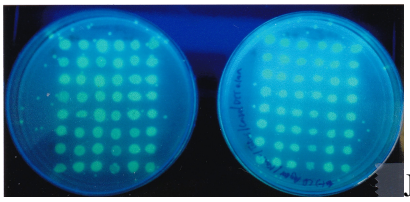
JM3 on GMML media. left: control/right: experiment



JM3 on common media. left: experiment/right: control



JW12N on GMML media. Left: experiment/right: control



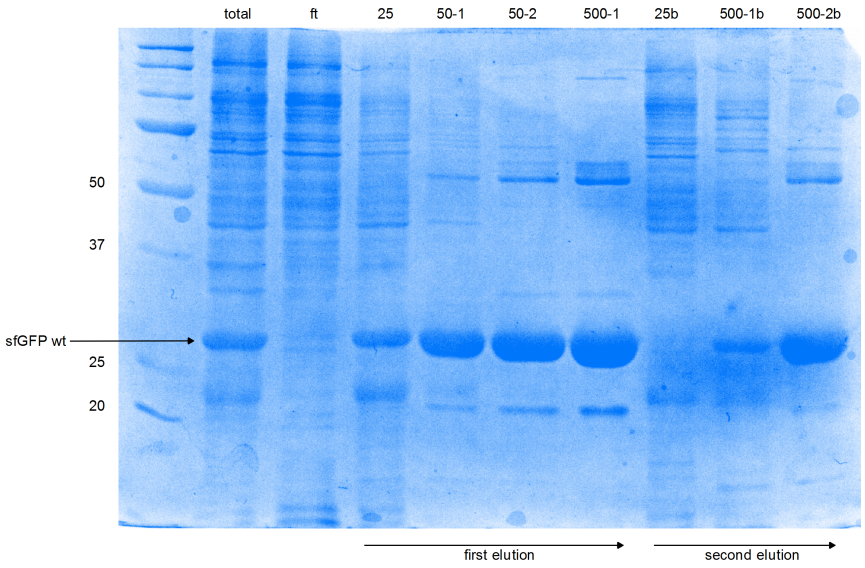
JW12N on common media. Left: experiment/right: control

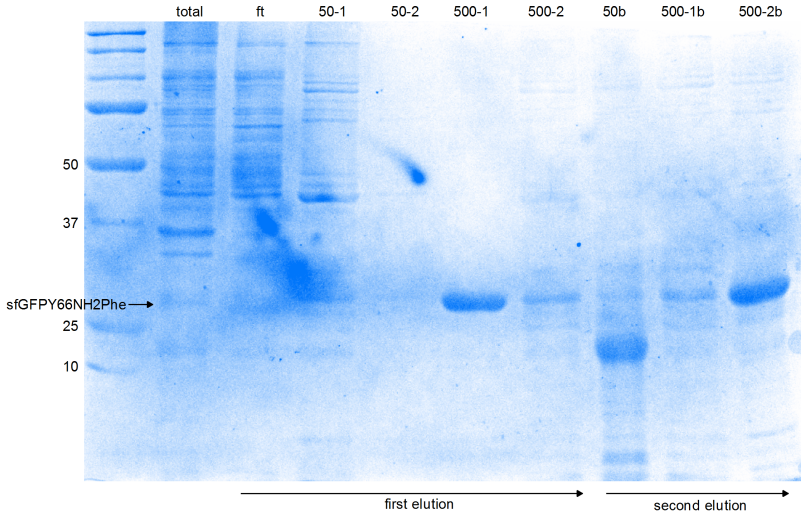
4. Protein purification of sfGFP mutant containing NH_2Phe:

After it was found that there was no obvious difference in fluorescence strength after cell growth with or without DiaPhe, I attempted to incorporate NH_2Phe into sfGFP as a control experiment. The purpose was to find out if unAA can be incorporated at Y66TAG position and see if the resulting sfGFP mutant can change its fluorescence in the presence of NO.

Genehog/pLei-sfGFP-Y66TAG/pBK-NH_2Phe or Genehog/pLei-sfGFP-Y66TAG was transferred into 5ml LB/5µl Cm/5µl Kan and incubated at 37℃ for 3h. Next, 200µl of each cell suspension was transferred into 2ml LB/200µl Kan/200µl Cm with or without NH_2Phe and incubated under

37℃ for 2 days. The cells were centrifuged then washed with water and centrifuged again. The pellet was resuspended in binding buffer and sonicated. After the sonication, protein purification was done following the standard protocol:[36]





I was glad to observe that the incorporation of NH_2Phe was successful.

5. Transformation:

   After the incorporation of NH_2Phe was confirmed, the next step was to transfer pLei-sfGFP-Y66TAG into a new cell line and to examine unAA incorporation with correspond tyrosyl tRNA/synthetase pair.

   Plates were made for transformation:

Plates	1	2
Agar (ml)	50	50
Cm_{34} (μl)	74	74
Kan_{50} (μl)	50	0
Ap_{30} (μl)	0	150

   a. the following plasmids were transferred into cells:

Number	Plasmid	Cell line
1	pLei-sfGFP-Y66TAG	Genehog
	pBK-NH_2Phe	
2	pLei-sfGFP-Y66TAG	Genehog
	pBK-STyr	
3	PET22b-sfGFP-Y66TAG	BL21(DE3)
	pEvol-DiaPhe	

   b. The cell suspensions were incubated under 37℃ for 1h. Then cells were spread on the plate and incubated at 37℃ for 1 day. On the next day, single colonies from 1 and 2 were picked into 2.5ml LB/Cm, Kan and incubated for 1h; transferred 50μl of 1 and 100μl of 2 to 6ml LB/Cm, Kan. Six colonies of 3 were picked into 6ml LB/Ap, Cm and then incubated for 1h; the medias were kept for further induction.

   c. 1 and 3 from media (OD: 0.4 – 0.5) were induced as following:

cells	workout	IPTG (100mM)	Ara (20%)	UAA (100mM)

(1) 6ml Genehog/sfGFP-Y66TAG/NH_2Phe	2ml (-)non-ind	0	0	0
	2ml (+)IPTG (0.2mM)/Ara (0.4%)	4 μl	40 μl	0
	2ml (+/+) IPTG (0.2mM)/Ara (0.4%)/NH_2Phe (2mM)	4 μl	40 μl	40 μl
(3) 6ml BL21(DE3)/sfGFP-Y66TAG/DiaPhe	2ml (-)non-ind	0	0	0
	2ml (+)IPTG (0.2mM)/Ara (0.4%)	4 μl	40 μl	0
	2ml (+/+) IPTG (0.2mM)/Ara (0.4%)/DiaPhe (2mM)	4 μl	40 μl	40 μl

After induction, the cells were incubated at 37 ℃ for 18h. Samples were divided equally: 1ml of each sample for SDS-PAGE analysis, 1ml for fluorescence and OD scan.

d. Fluorescence analysis: 1ml cells was collected, washed, and resuspended in 1ml PBS. Centrifuged one more time and then supernatant was discarded, the pullet was resuspended by 1ml PBS. The result showed below:

Cell	Emission (nm)	Excitation (nm)
(1) (+)IPTG	508	454
(+/+)IPTG + NH_2Phe	500	454
(3) (+)IPTG	508	486
(+/+)IPTG + DiaPhe	508	484

Fluorescence intensity:

Cell	Workout	Fluorescence intensity
(1)		Ex/em: 454/508
	(-)	709
	(+)	896
	(+/+)	2308
	blank	555
(3)		Ex/em: 484/520*
	(-)	551
	(+)	89076
	(+/+)	22782
	Blank	437

*the excitation and emission wavelength were too close to set (484nm and 508nm). The closest was 484nm and 520nm.

Then the OD value was measured: OD_{600} test was in transparent plate, containing 120 µl PBS and 30 µl cell culture.

Cell line	Workout	OD_{600} value	OD_{600} (corrected)
(1)	(-)	0.158	0.393
	(+)	0.152	0.391
	(+/+)	0.163	0.417
	blank	0.038	0.085
(3)	(-)	0.264	0.662
	(+)	0.454	0.707
	(+/+)	0.421	1.614

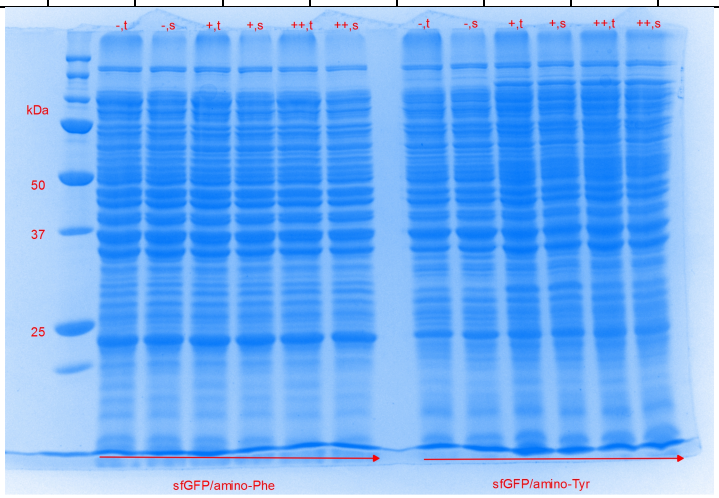
Fluorescence divided by OD_{600}:

Cell line	workout	①Fluorescence – blank (454,500)	②5 X (OD_{600} – blank)	①/②
(1)	(-)	188	1.54	122.1
	(+)	413	1.53	270.0
	(+/+)	2306	1.66	1389.2
		①Fluorescence – blank (484,520)	②5 X (OD_{600} – blank)	
(3)	(-)	114	2.89	39.4
	(+)	88639	8.31	10666.5
	(+/+)	22349	7.65	2920.9

e. Protein analysis on SDS-PAGE:

1ml media was used to resuspend cells, then the cell suspension was centrifuged and the supernatant was discarded. 100µl BugBuster was added to lyse the cell. Then centrifuge was done at max speed for 20min under 4℃. SDS-PAGE was performed following the standard protocol, the result showing below:

lane	(1)						(3)					
1	2	3	4	5	6	7	8	9	10	11	12	13
marker	-t	-s	+t	+s	++t	++s	-t	-s	+t	+s	++t	++s



t: total protein; s: soluble protein; amino-Try: DiaPhe

6. Protein and fluorescence analysis of (2) and (3) from experiment 5:

   a. Cell induction:

   4-6 colonies were picked up from experiment 4 in LB media:

   (1) Genehog/pLei-sfGFP-Y66TAG/pbk-STry to 6ml LB/Kan, Cm;

   (2) BL21(DE3)/pET22b-sfGFP-Y66TAG/pEvol-DiaPhe to 6ml LB/Ap, Cm.

   The medias were incubated under 37℃ until OD_{600} value reached 0.3 - 0.4.

   b. Induction:

   6ml Genehog/pLei-sfGFP-Y66TAG/pBK-sTry:

Workout	100mM IPTG	200mM sTry
2ml non-ind. (-)	0	0
2ml IPTG (0.2mM) (+)	4µl	0
2ml IPTG (0.2mM), sTry (1mM) (+/+)	4µl	100µl

6ml BL21(DE3)/pET22b-sfGFP-Y66TAG/pEvol-DiaPhe:

workout	100mM IPTG	20% Ara	100mM DTT	100mM DiaPhe
2ml non-ind. (-)	0	0	0	0
2ml IPTG (0.1mM), Ara (0.4%), DTT (0.1mM) (+)	2 μl	40 μl	2 μl	0
2ml IPTG (0.1mM), Ara (0.4%), DTT (0.1mM), DiaPhe (1mM) (+)	2 μl	40 μl	2 μl	20 μl

Cultures of (2) were incubated under 37 ℃ for 21 h. Cultures of (3) were incubated under 37 ℃ for 18 h. After incubation, OD and fluorescence for cultures of (2) and (3) were determined:

Emission and excitation scan for (3) (+) and (+/+):

Workout	ε_{mmax} (nm)	ε_{xmax} (nm)*
(+)	508	486
(+/+)	510	488

*signal in graph was rising from 350 – 490nm when ε_m was 518nm.

Workout	OD_{600}	Fluo (ε_x: 488nm, ε_m: 520nm)	Fluo – blank/ 5(OD – blank)
(-)	0.873	957	148.6
(+)	1.779	24669	2871.9
(+/+)	1.891	27503	3007.8
Blank	0.087	373	NA

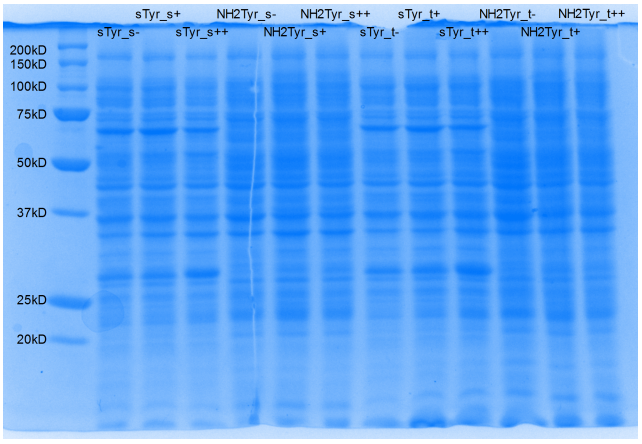
Emission and excitation scan for (2) (+) and (+/+):

Workout	ε_{mmax} (nm)	ε_{xmax} (nm)**
(+)	526	442
(+/+)	520	438

**signal in graph was like a "M" shape when ε_m was 520nm, the strongest excitation wavelength was at 360nm and 438nm.

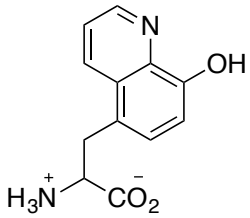
Workout	OD_{600}	Fluo (εx: 438, ε_m: 520)	Fluo (εx: 360, ε_m: 520)
(-)	0.352	898	466
(+)	0.309	835	537
(+/+)	0.448	874	467
Blank	0.087	859	418

Ran SDS-PAGE:



7. Transferred pEvol-HQAla into cell:

   Since HQAla had a similar structure to DiaPhe, next I attempted to see if this tyrosyl tRNA/synthetase pair could recognize DiaPhe.



HQala

   a. Transformation:

plasmid	Cell line
pET22b-sfGFPY66TAG	BL21(DE3)
pEvol-HQAla	

Plate: Agar 25ml, cm 25µl, Ap 25µl.

   b. On the next day, cells were induced:

Workout	100mM DTT	20% Ara	100mM IPTG	UAA
(-) blank	0	0	0	0
(+) DTT (0.2mM)/IPTG (0.1mM)/Ara (0.4%)	4µl	40µl	2µl	0
(+/+) DTT (0.2mM)/IPTG (0.1mM)/Ara (0.4%)/DiaPhe (2mM)*	4µl	40µl	2µl	0.91mg
(+/+) DTT (0.2mM)/IPTG (0.1mM)/Ara	4µl	40µl	2µl	1.27mg

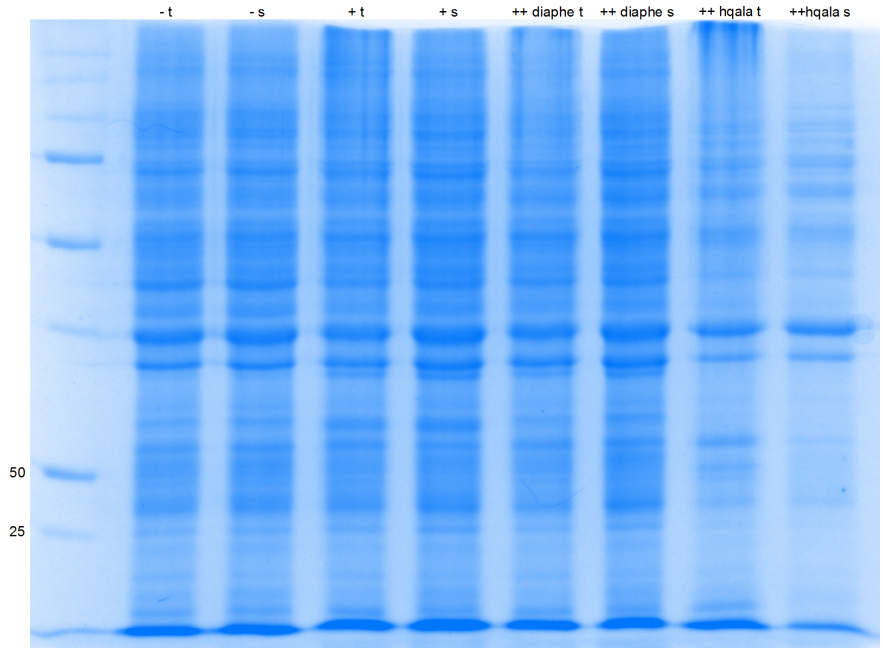
(0.4%)/HQAla (2mM)				

*in this experiment, this cell was from previous experiment.

c. On the next day, fluorescence and OD_{600} were determined:

	-	+	+/+ DiaPhe	+/+ HQAla
OD – blank	0.801	0.881	0.733	0.129
ε_m – blank	2936	10513	4317	2699
peak	516 nm	512 nm	512 nm	640 nm
ε_x – blank	26814	35814	7154	590
peak	420 nm	486 nm	484 nm	410 nm

d. Ran SDS-PAGE:



8. Selection of tRNA synthetase:

Then I examined other libraries for the incorporation of DiaPhe. It was followed by the instruction from Dr. Schultz.[24]

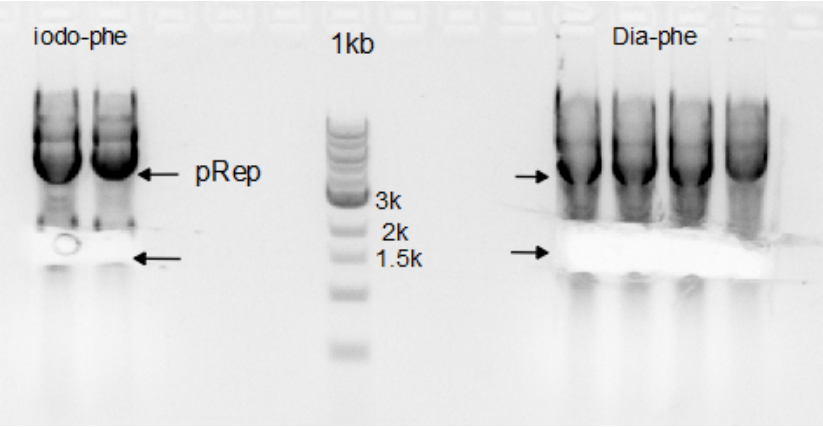
a. A new library (pBK-TyrRS-WN1-lib) was prepared and transferred in host cells (Dh10B/pRep-TyrT) by Dr. Niu. The cell was induced in 55ml LB media (Kan 55µl, Tet

137µl). IodoPhe was used in this experiment as a positive control. On the next day, the OD_{600} value was determined to be 2.2.

b. Made plates for experiment:

workout	LB/Agar (ml)	Kan (µl)	Cm (µl)	Tet (µl)	DTT (mM)	DiaPhe (mM)	IodoPhe (mM)
(+)	50	50	74	125	0.2	2	0
(-)	50	50	74	125	0.2	0	0
Control	50	50	74	125	0.2	0	2

c. 45µl cell suspension and 155µl water were transferred on each plate. Plates were induced under 37℃ overnight. On the next day, the cells were pooled by 2 ml X 3 LB. The cells were centrifuged and the supernatant was discarded. Mini-prep was done to get the plasmid of library. Then DNA gel was performed to purify the plasmid following standard protocol.[37] The DNA concentration was then determined: DiaPhe 255ng/µl (5µl), IodoPhe 244ng/µl (6µl).

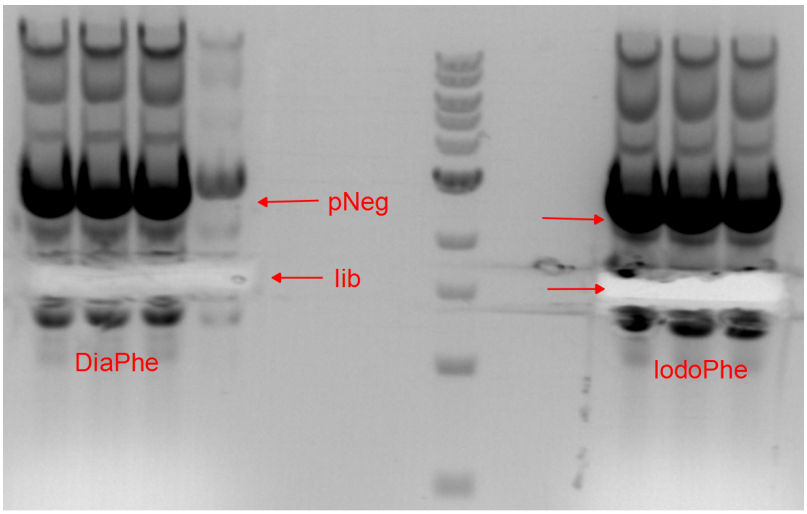


After plasmid was obtained, the electroporation of pNeg was conducted and then the plasmid of library was transferred into the cell. The cells were recovered in SOC medium

for 1h under 37℃. After the recovery, 50µl media was transferred into 50ml LB media (with Kan_{50} and $Tet_{12.5}$) and incubated under 37℃ overnight.

d. On the next day, the OD_{600} value were determined: DiaPhe lib 2.4; IodoPhe lib 2.5. Then plates for negative selection were made: Lb/Agar 50ml, Kan_{50}, Ap_{50} and 0.2% Ara. Then transferred 45µl pNeg/pBK-DiaPhe-lib and 155µl water on one plate, 40µl pNeg/pBK-IodoPhe-lib and 160µl water on another plate. Both plates were induced under 37 ℃ overnight.

e. On the next day, the cells were grown well. The cells were pooled and mini-Prep was done to get the plasmid. DNA gel was performed to purify the plasmid:



I next determined the plasmid concentration: DiaPhe-lib 244ng/µl (8µl), IodoPhe-lib 158ng/µl (8µl). 2µl of plasmids was used to transfer into chemically competent DH10B cell with pRep-tRNAase plasmid, the rest of plasmids were stored at -80℃.

f. Made plates for third round positive selection:

workout	Lb/Agar	Kan (μl)	Cm (μl)	Tet (μl)	DTT (mM)	UAA (mM)
1	50ml	50	74	125	2	DiaPhe 2
2	50ml	50	74	125	2	IodoPhe 2

150μl water and 10μl cell suspension was plated on each plate and incubated under 37 ℃ overnight. On the next day, checked the colonies on plates: there were 2240 colonies, 2 colonies with fluorescence on the plate with DiaPhe; there were 600 colonies, 1 colony with fluorescence on the plate with IodoPhe. Media was made for these 3 colonies: LB 5ml, Kan 5μl, Tet 12.5μl. These 3 colonies were transferred separately in media, incubated under 37℃ overnight. On the next day, 750μl of each media was taken into 50% glycerol solution to make glycerol stock, stored at -80℃ for further experiment.

9. After 3 rounds of selection, it was tried to express sfGFP-DiaPhe from the previous cell line.
   a. 50μl stock solution of BL21(DE3)/pET22b-sfGFPY66TGA/pEol-DiaPhe was transferred into 8ml LB/8μl Ap/8μl Cm and incubated under 37℃ for 3h. Then the cells were induced:

workout	100mM IPTG	20% Ara	100mM DiaPhe	100mM DTT
(-)	0	0	0	0
(+)	2 μl	40 μl	0	0
(+/+) without dTT	2 μl	40 μl	2 μl	0
(+/+) w/ DTT	2 μl	40 μl	2 μl	20 μl

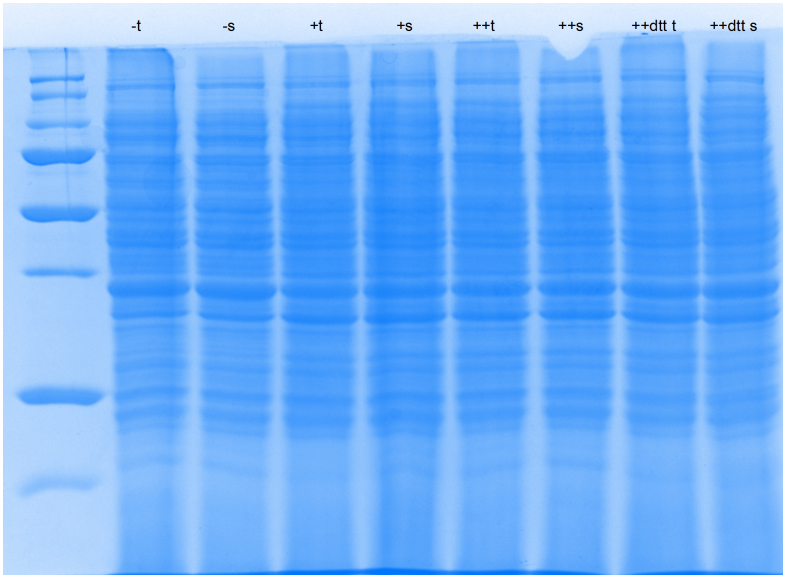
For two groups of (+/+) with or without DTT, DiaPhe and DTT (if with DTT) were added into the media, incubated under 37℃ for 15min, then added IPTG and Ara. Incubated under 37℃ overnight.

b. On the next day, I detected the OD value and fluorescence:

1ml media was taken, centrifuged and the supernatant was discarded, the pellet was resuspended by 1ml PBS, repeated one more time. 30 µl suspension and 120 µl PBS for OD and 150 µl suspension were taken for fluorescence:

workout	blank	+	+/+	+/+ DTT	
fluorescence	945	35412	37201	36184	Emission
	490nm	510nm	510nm	510nm	
	NA	59353	64276	63223	Excitation
	NA	486nm	490nm	488nm	
OD_{600} (5X dilution)	0.129	0.363	0.333	0.333	
(Fluo – blank)/5(OD – blank)	NA	29549	35545	34548	

c. Ran protein gel:



10. Then it was attempted to purify sfGFP-NH_2Phe as a control:

50 µl stock media of genehog/pLei-sfGFPY66TAG/pBK-NH_2Phe was taken into 2ml LB/ 2 µl Cm/2 µl Kan and incubated under 37℃ overnight. On the next day, 50 µl media was

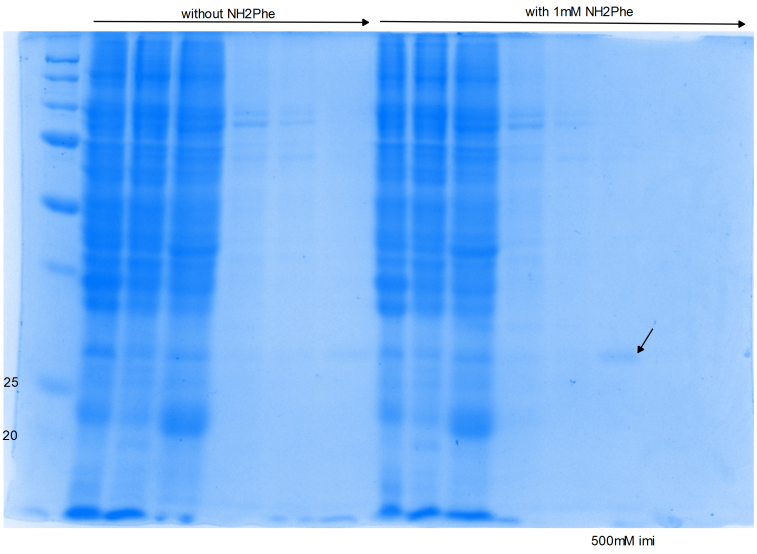
transferred to each: 2ml LB/ 2 µl Cm/ 2 µl Kan X 5, incubated under 37℃ for 3h. Then induced the cells:

Workout	100mM IPTG	100mM NH_2Phe
(-) non-ind.	0	0
(+)IPTG (0.2mM)	4 µl	0
(+/+)IPTG (0.2mM) / NH_2Phe (1mM)	4 µl	20 µl
(+/+)IPTG (0.2mM) / NH_2Phe (2mM)	4 µl	40 µl
(+/+)IPTG (0.2mM) / NH_2Phe (5mM)	4 µl	100 µl

After the induction, the cells were incubated under 37℃ overnight. On the next day, fluorescence and OD_{600} were measured:

Workout	-	+	+/+ 1	+/+ 2	+/+ 5
(OD – blank)/5	0.600	0.512	0.483	0.488	0.500
ε_m - blank	1496	1357	2979	4464	8018
ε_x - blank	1418	1494	2670	4142	7405
ε_{xmax}	426nm	484nm	448nm	450nm	450nm

NH_2Phe-sfGFP was purified following the standard protocol:

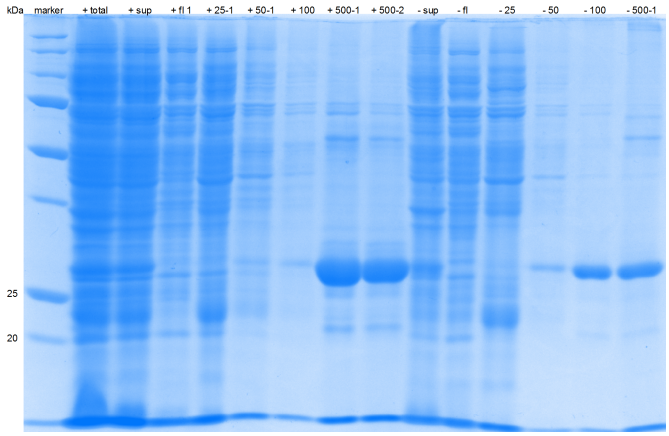


11. After the purification of sfGFP-NH_2Phe was performed, it was to try to purify DiaPhe -sfGFP-:

    50 µl stock media of BL21(DE3)/pET22b-sfGFPY66TAG/pEvol-DiaPhe was transferred into 2ml LB/2µl Cm/2µl Kan and incubated under 37℃ for 3h. Then the cells were induced:

Workout	100mM IPTG	20% Ara	100mM DTT	100mM DiaPhe
(-) IPTG (0.2mM)/Ara (0.4%)/DTT (0.1mM)	100 µl	1ml	50 µl	0
(+) IPTG (0.2mM)/Ara (0.4%)/DTT (0.1mM)/DiaPhe (1mM)	100 µl	1ml	50 µl	500 µl

    Then they were incubated under 37℃ overnight. On the next day, sfGFP-DiaPhe was purified following the standard protocol:



12. At last, it was tried to express sfGFP-HQAla as a verification of previous plasmid transformation and protein expression;

    a. Transformation: pEvol-HQAla and pET22b-sfGFP-Y66TAG were transferred into BL21(DE3). After transformation, it was incubated in 2ml LB/2 µl Ap/2 µl Cm under 37 ℃ for 3h. Then the cells were induced:

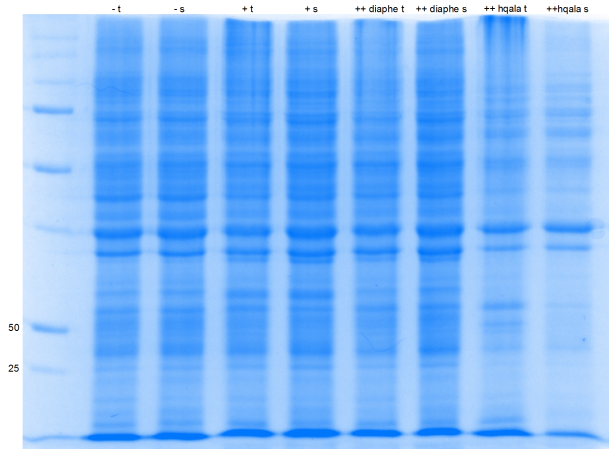
workout	100mM IPTG	100mM DTT	20% Ara	UAA
(-)	0	0	0	0
(+)0.2mM DTT/0.1mM	2 µl	4 µl	40 µl	0

IPTG/0.4%Ara				
(+/+)0.2mM DTT/0.1mM IPTG/0.4% Ara/2mM DiaPhe	2 µl	4 µl	40 µl	0.91mg
(+/+)0.2mM DTT/0.1mM IPTG/0.4% Ara/2mM HQAla	2µl	4µl	40µl	1.27mg

b. After in the induction, the cells were incubated under 37℃ overnight. On the next day, the OD value and fluorescence were measured:

workout	(-)	(+)	(+/+) w/ DiaPhe	(+/+) w/ HQAla
1/5 (OD- blank)	0.801	0.851	0.723	0.129
ε_m - blank	2936	10513	4317	2699
ε_m peak	516nm	512nm	512nm	640nm
ε_x - blank	26814	35814	7154	590
ε_x peak	420nm	486nm	484nm	410nm

c. Ran SDS-PAGE following the standard protocol:



Then the accident happened so that this research project had to be stopped.

CONCLUSION AND FUTURE PLAN

Unfortunately, my project had to be stopped midway because of my accident. Prior to the accident, I concluded that there was reactivity of the di-amine group with NO. I also proved that the DiaPhe was successfully incorporated as the desired unAA but I could not repeat the experiment to verify its incorporation into GFP. I could not prove if modified GFP was reactive with NO *in vivo*.

In the future, one may keep trying new mutagenesis to optimize new libraries of tyrosyl tRNA/synthetase pair, which can successfully recognize DiaPhe and incorporate it into GFP.

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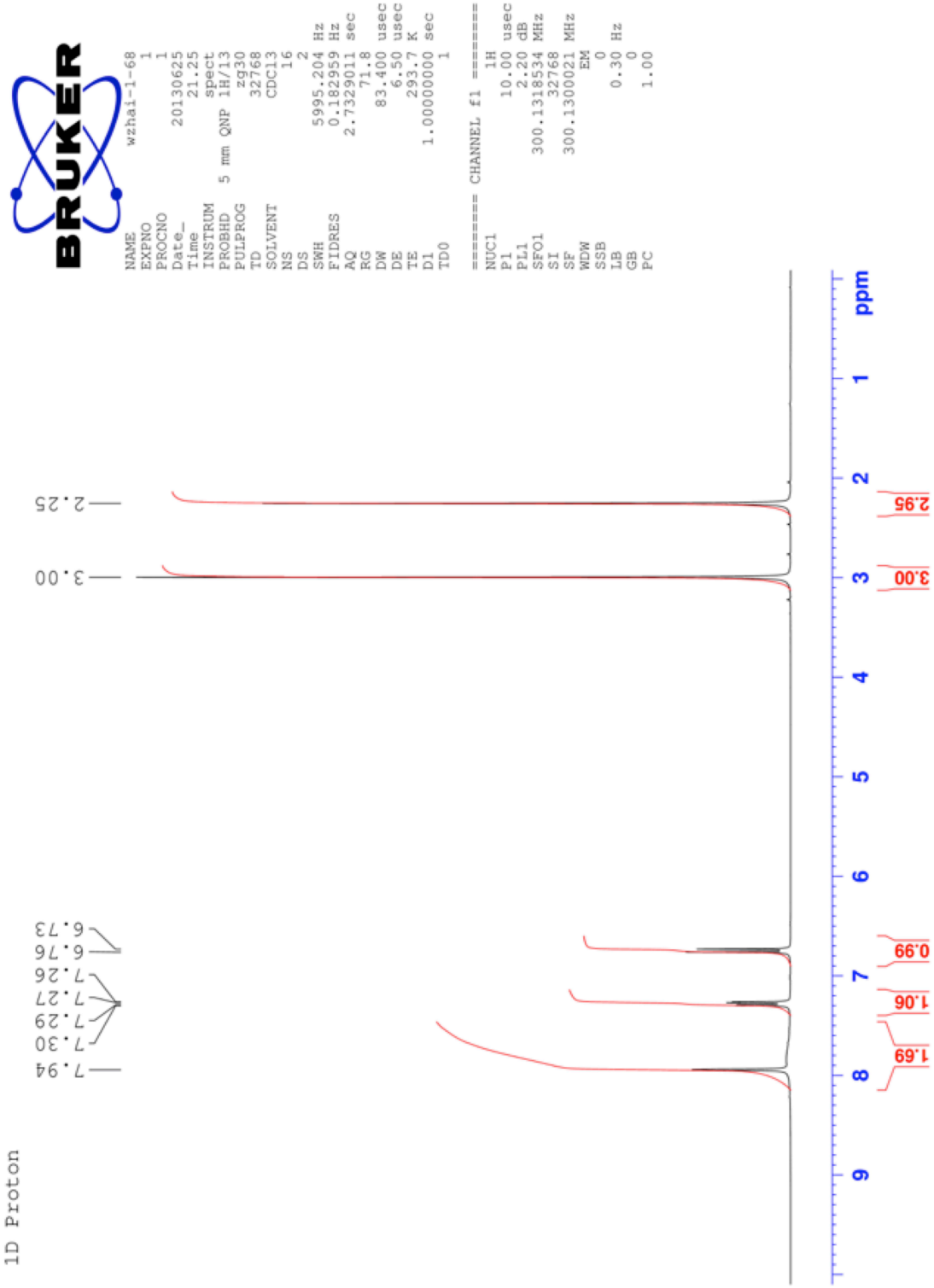
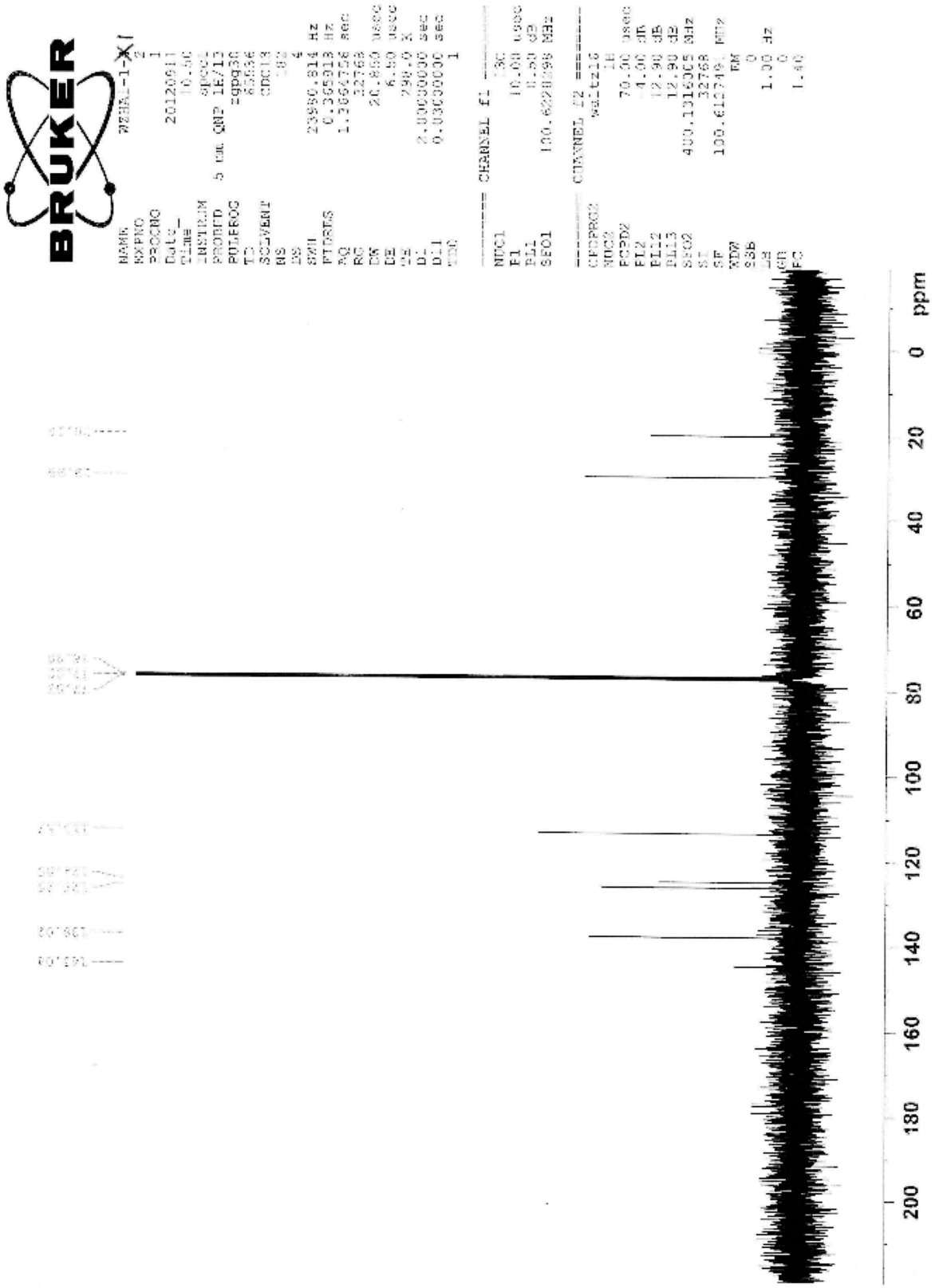
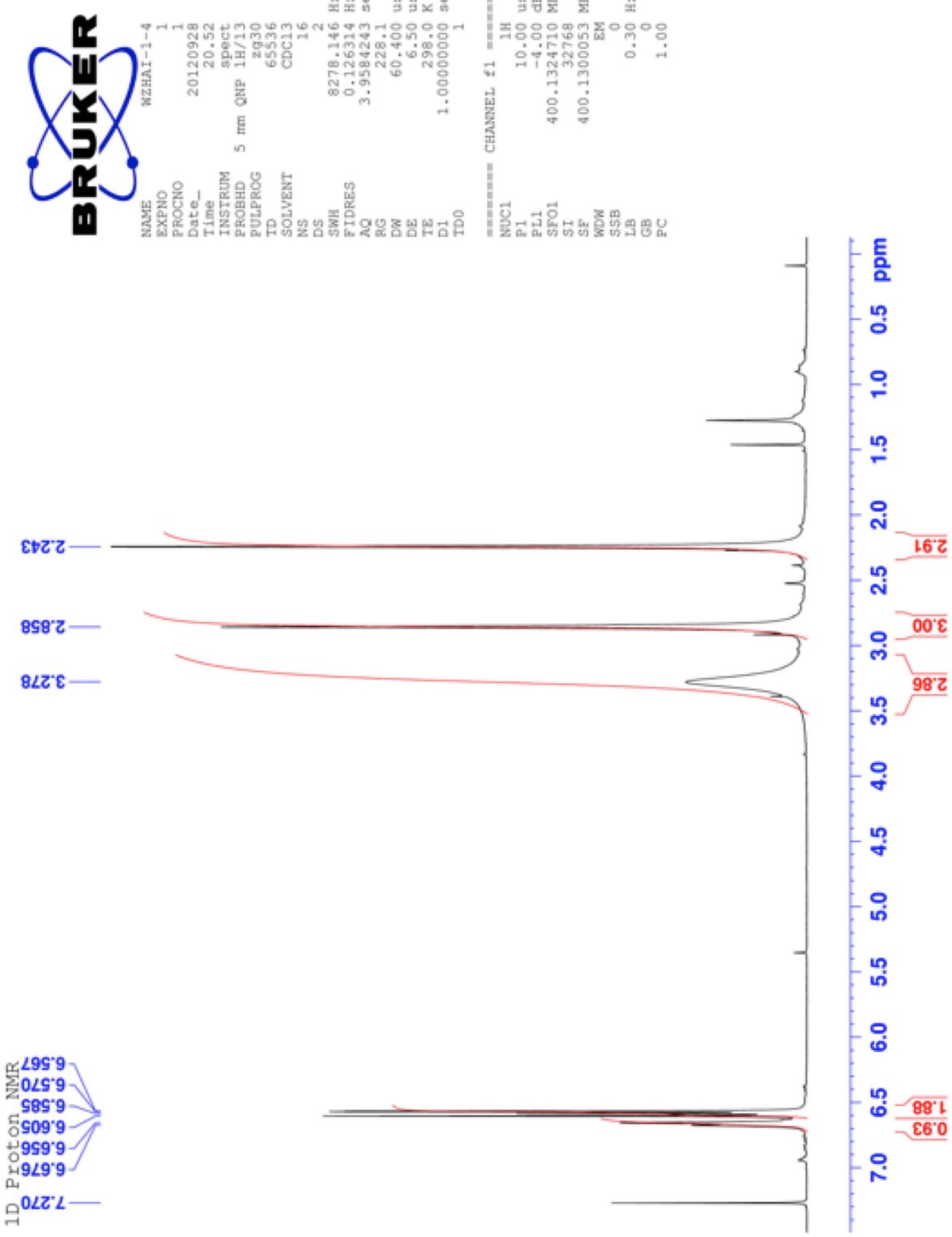
APPENDIX



H^1 NMR Spectra of compound **1**



C^{13} NMR Spectra of compound **1**



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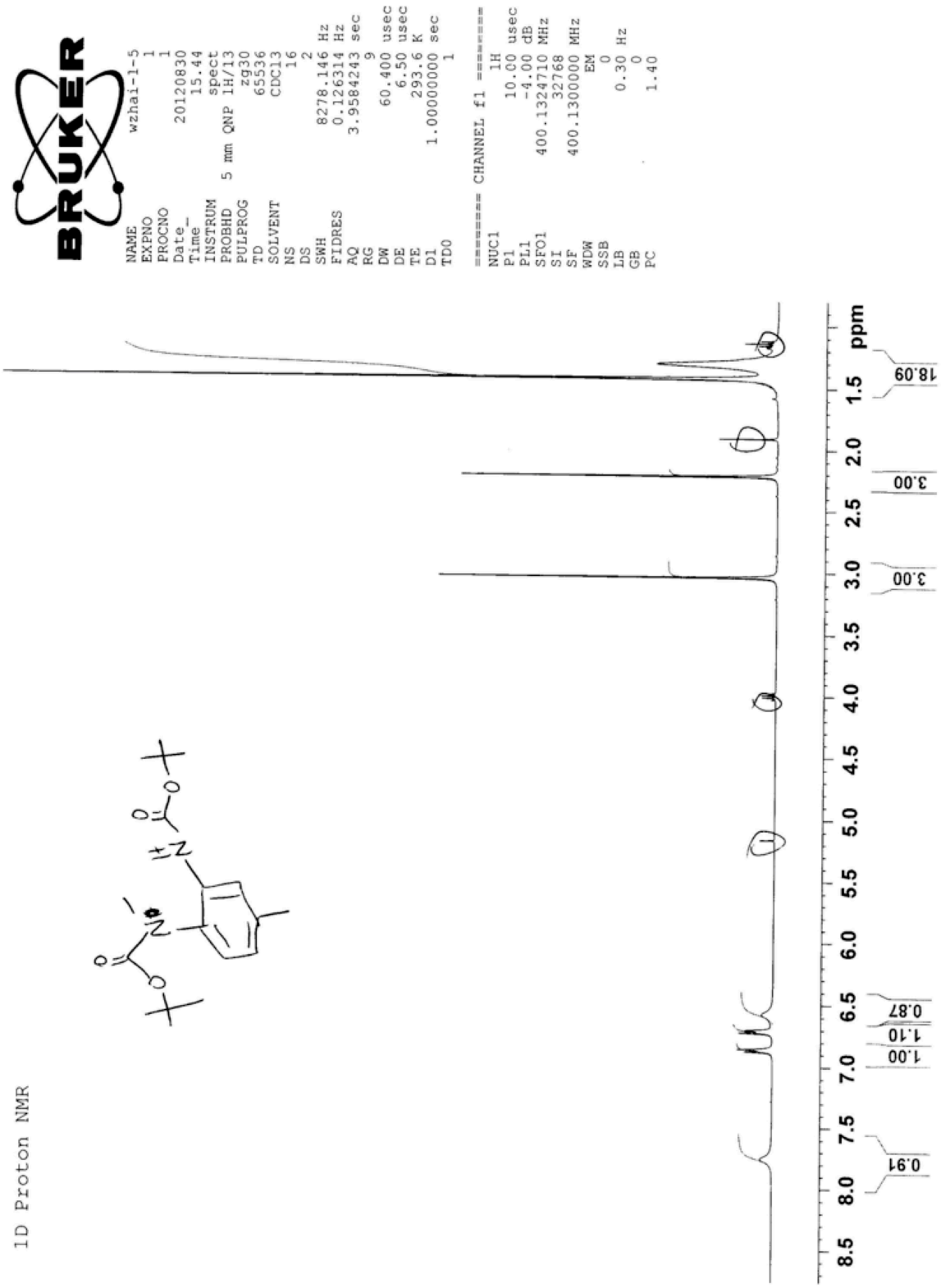
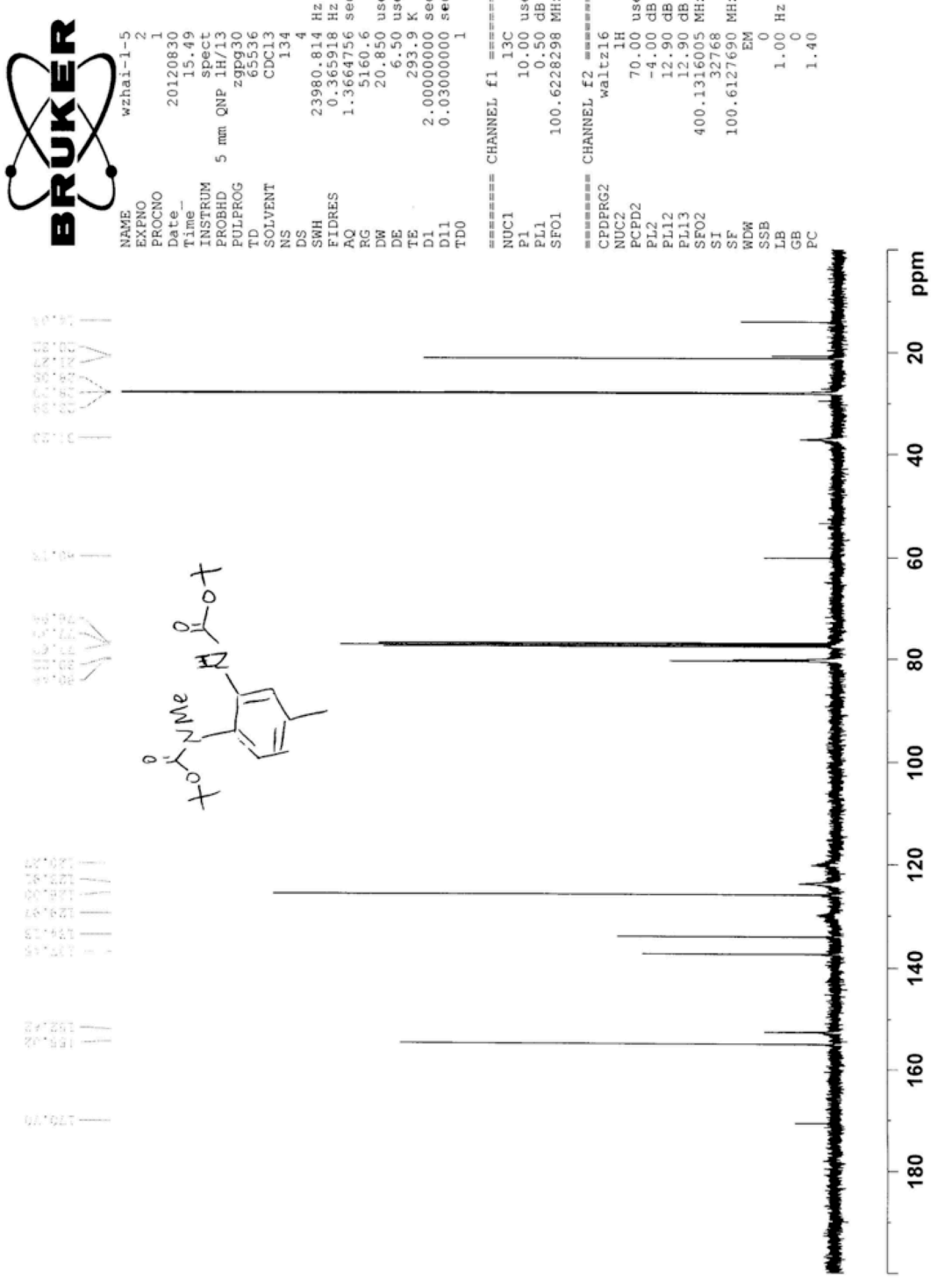


H^1 NMR Spectra of compound **3**



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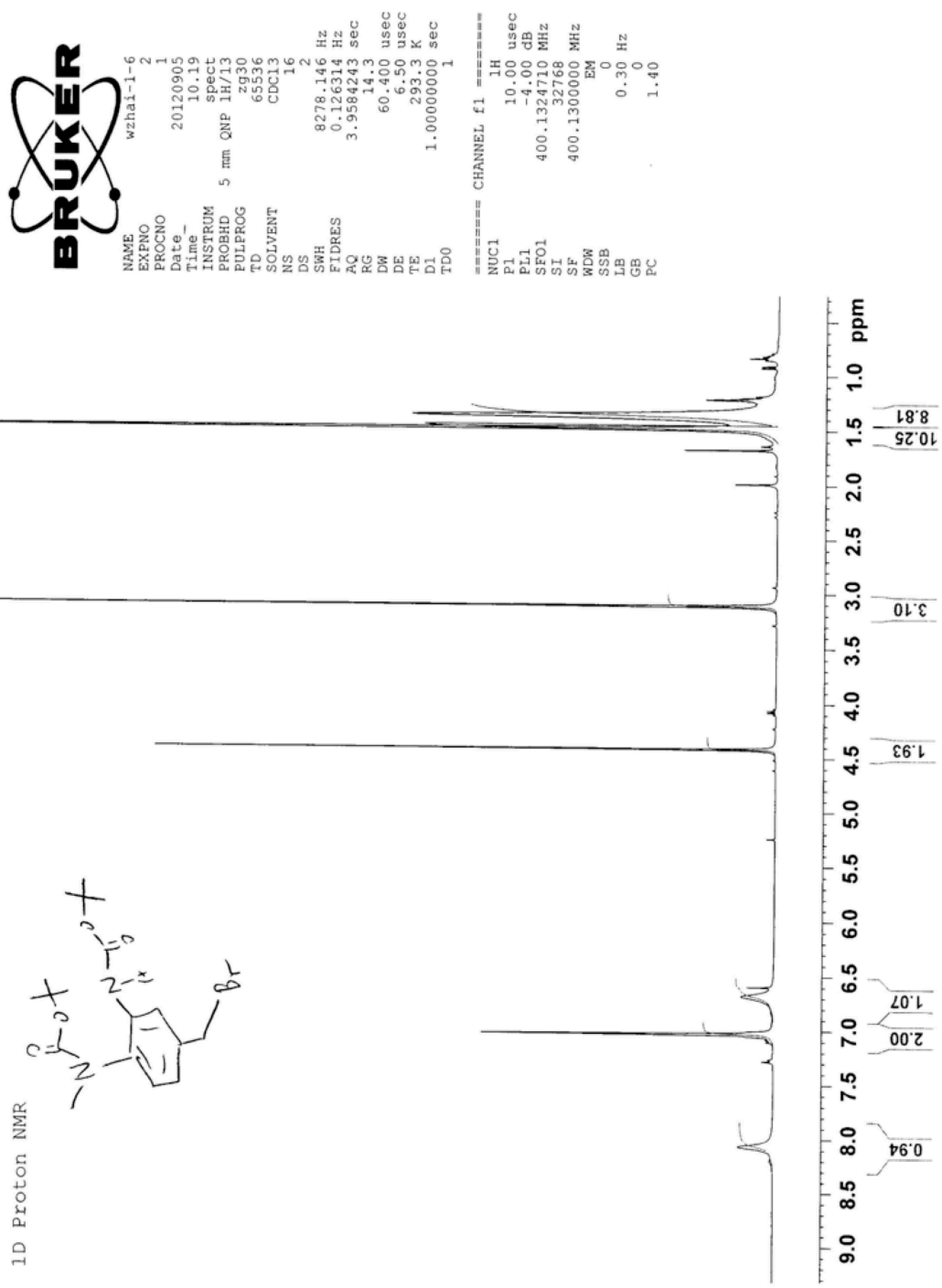

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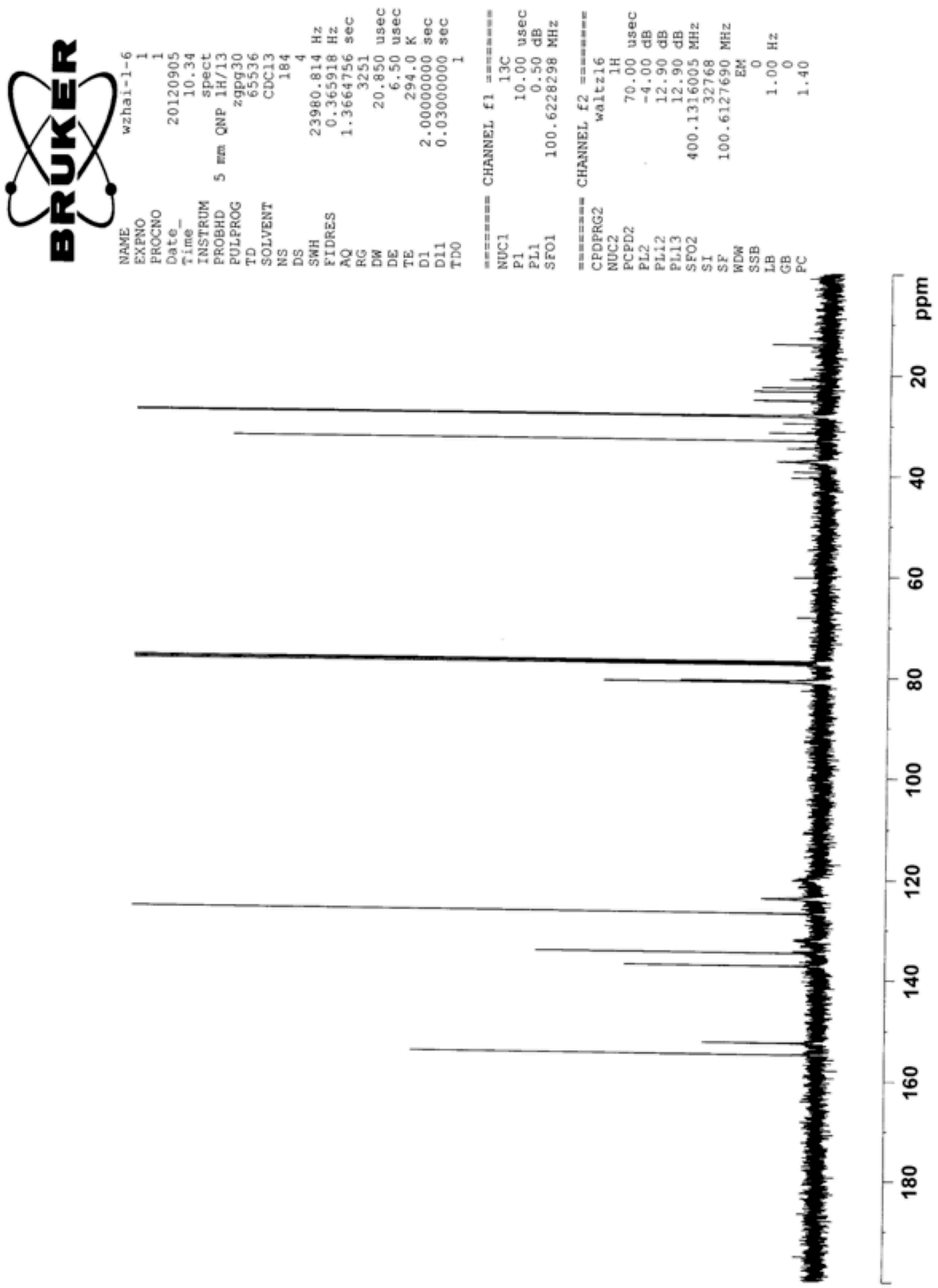

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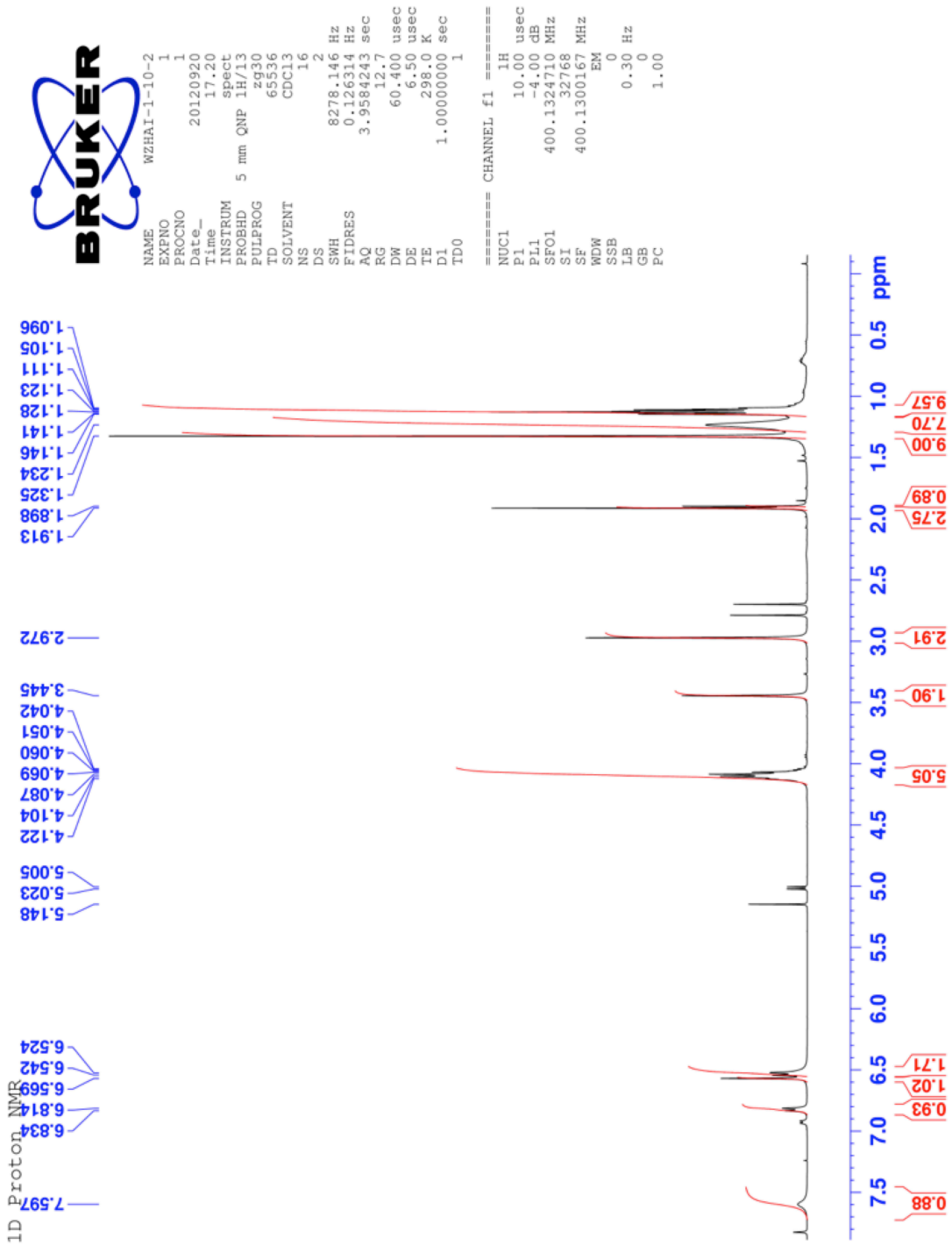
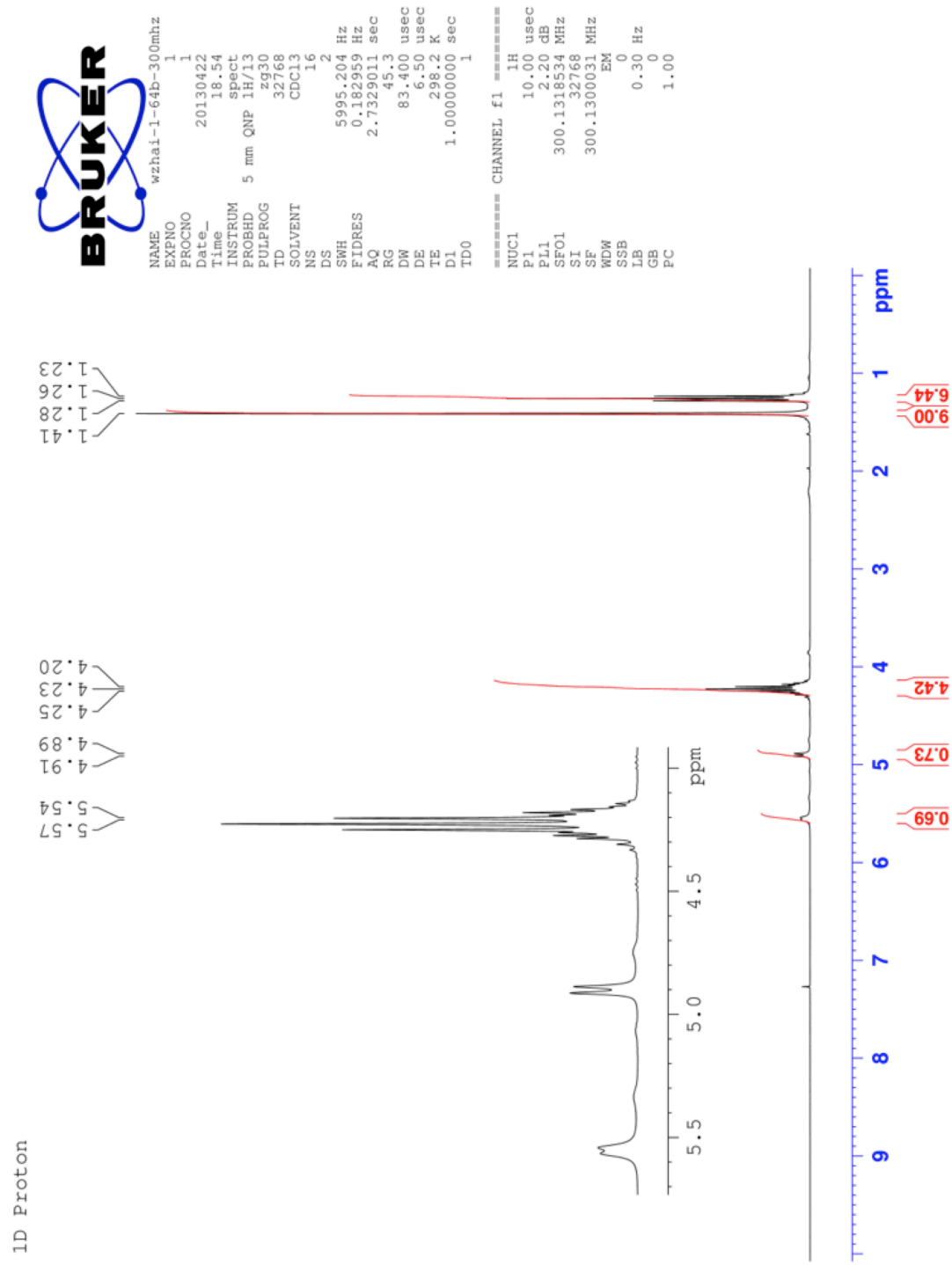


H^1 NMR Spectra of compound **5**

C^{13} NMR Spectra of compound **5**



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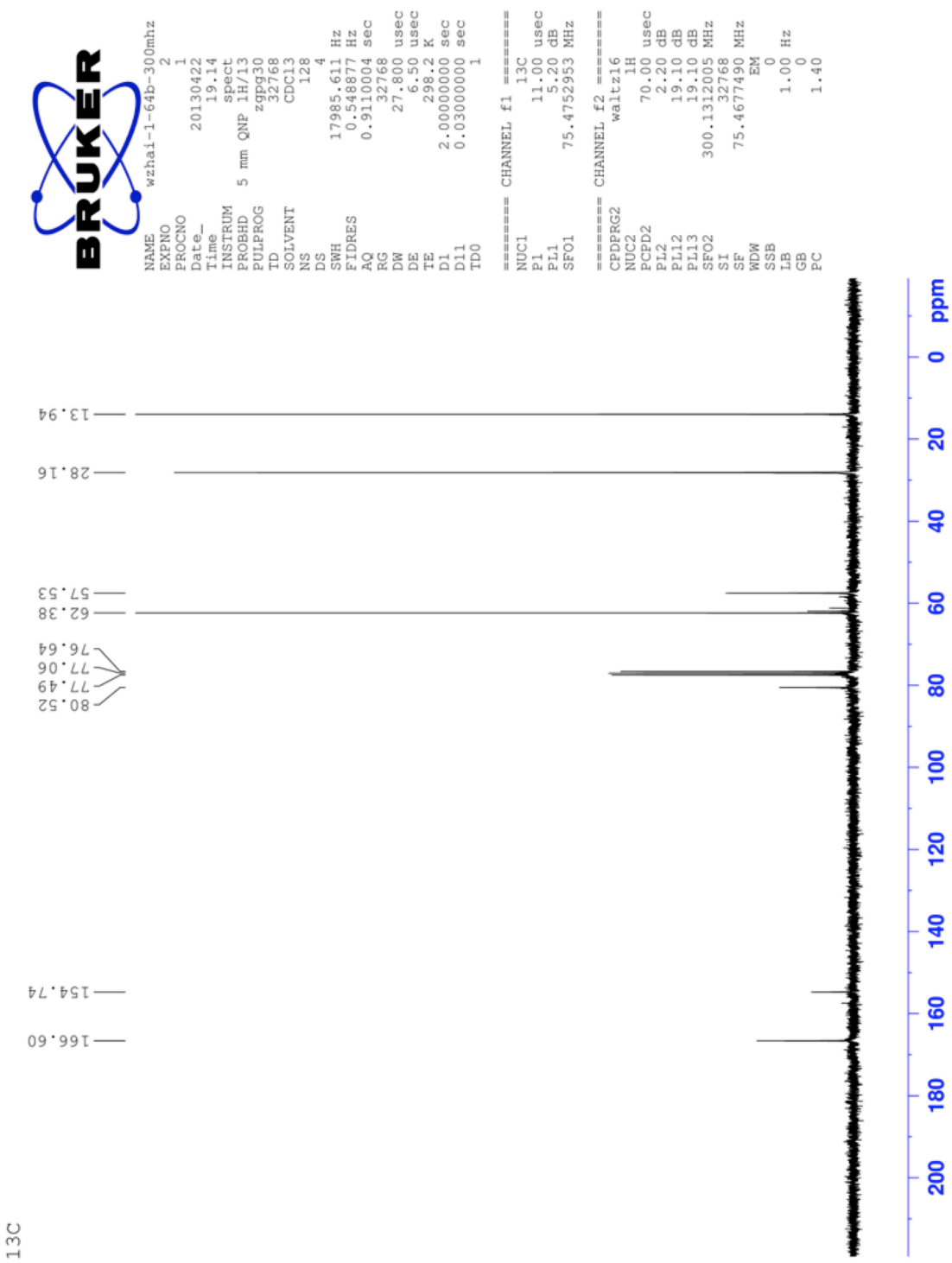

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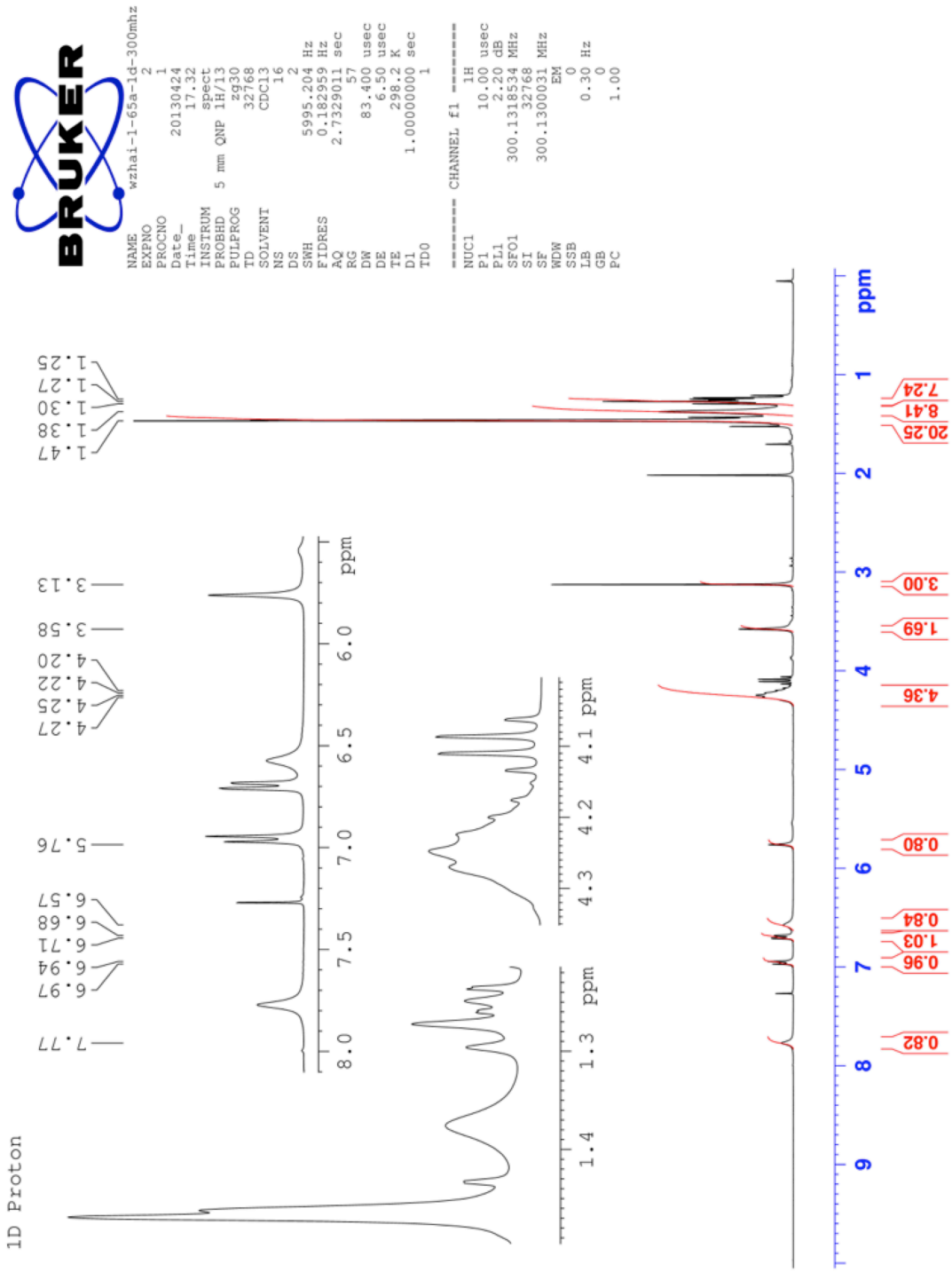

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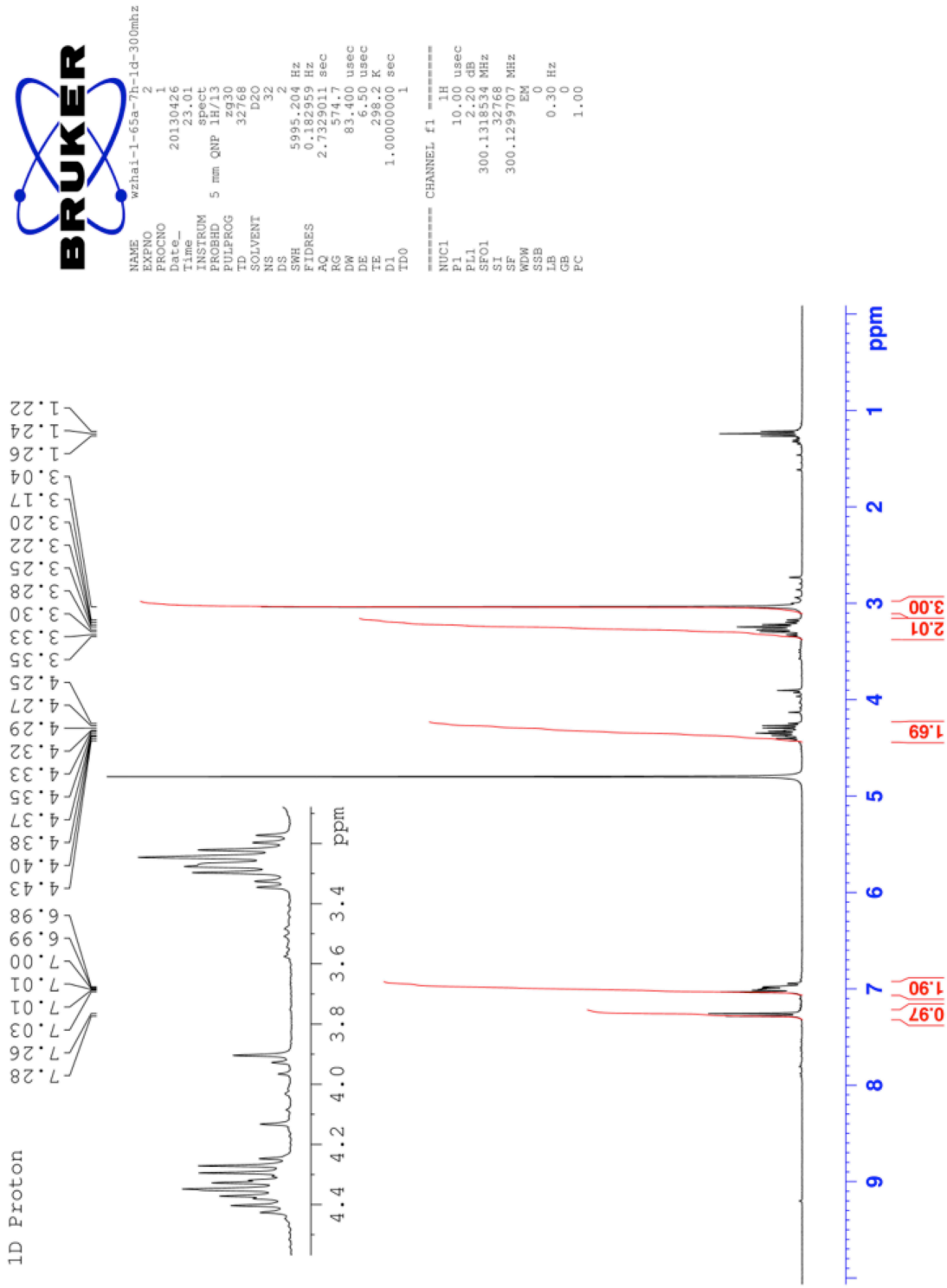

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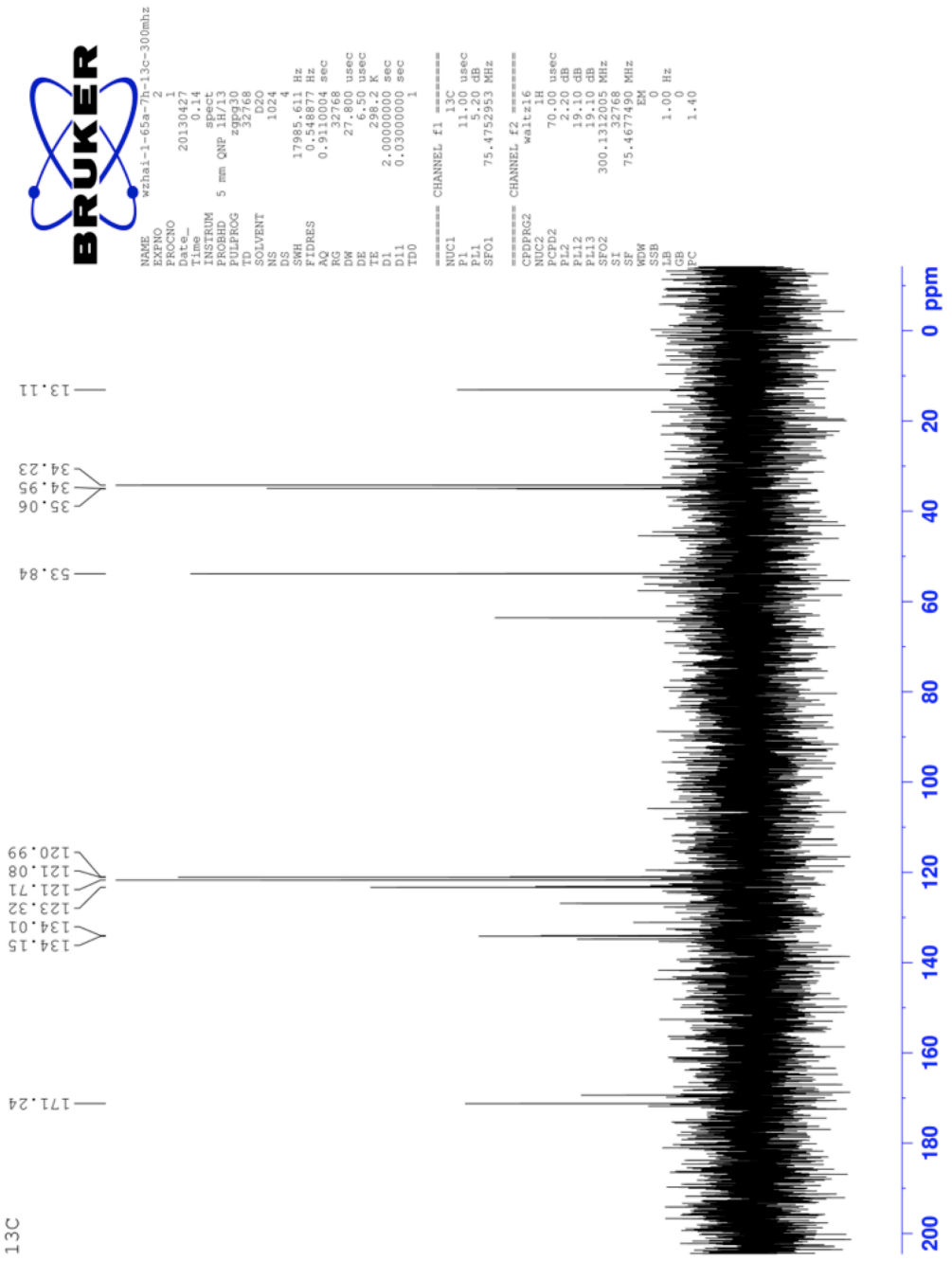
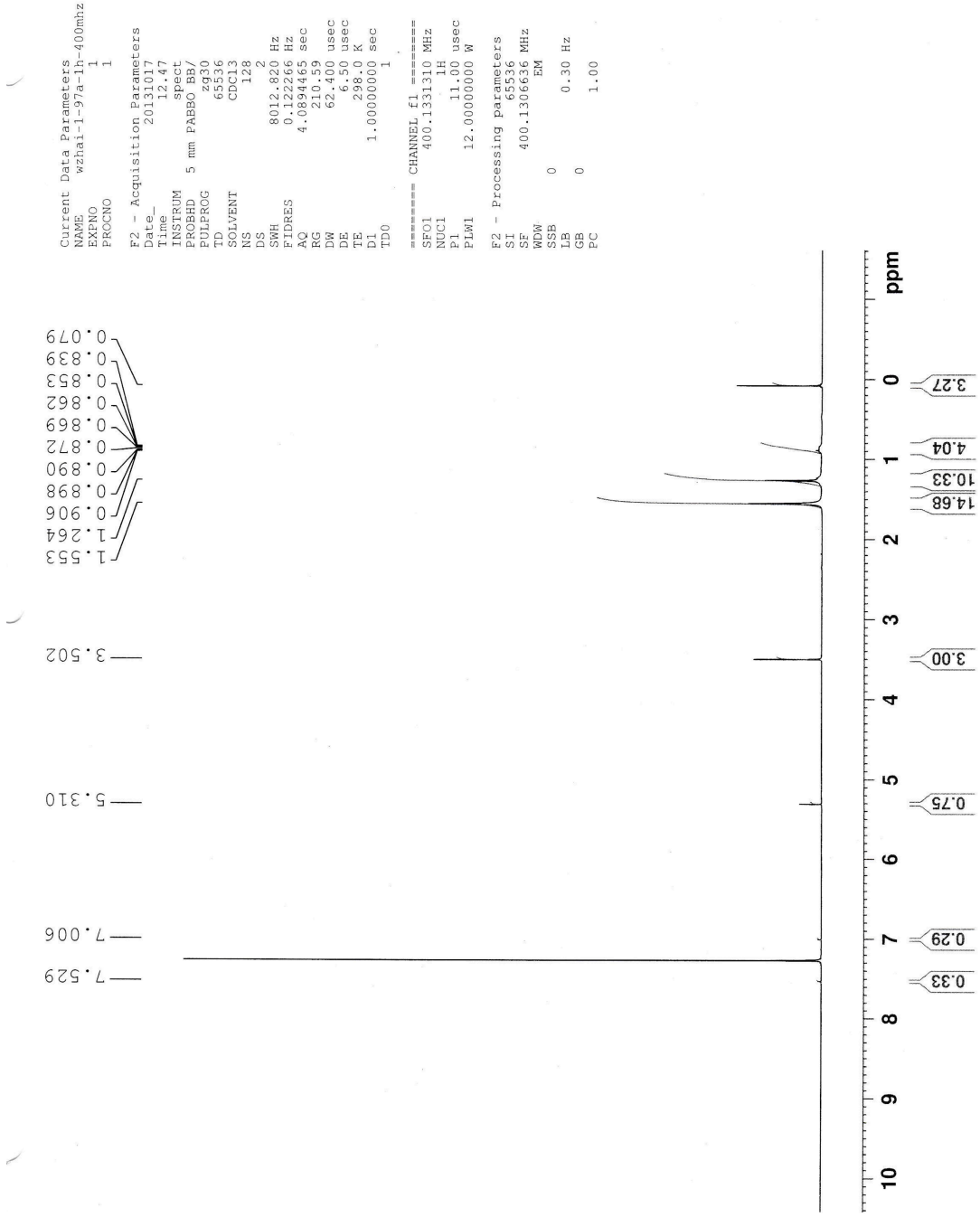
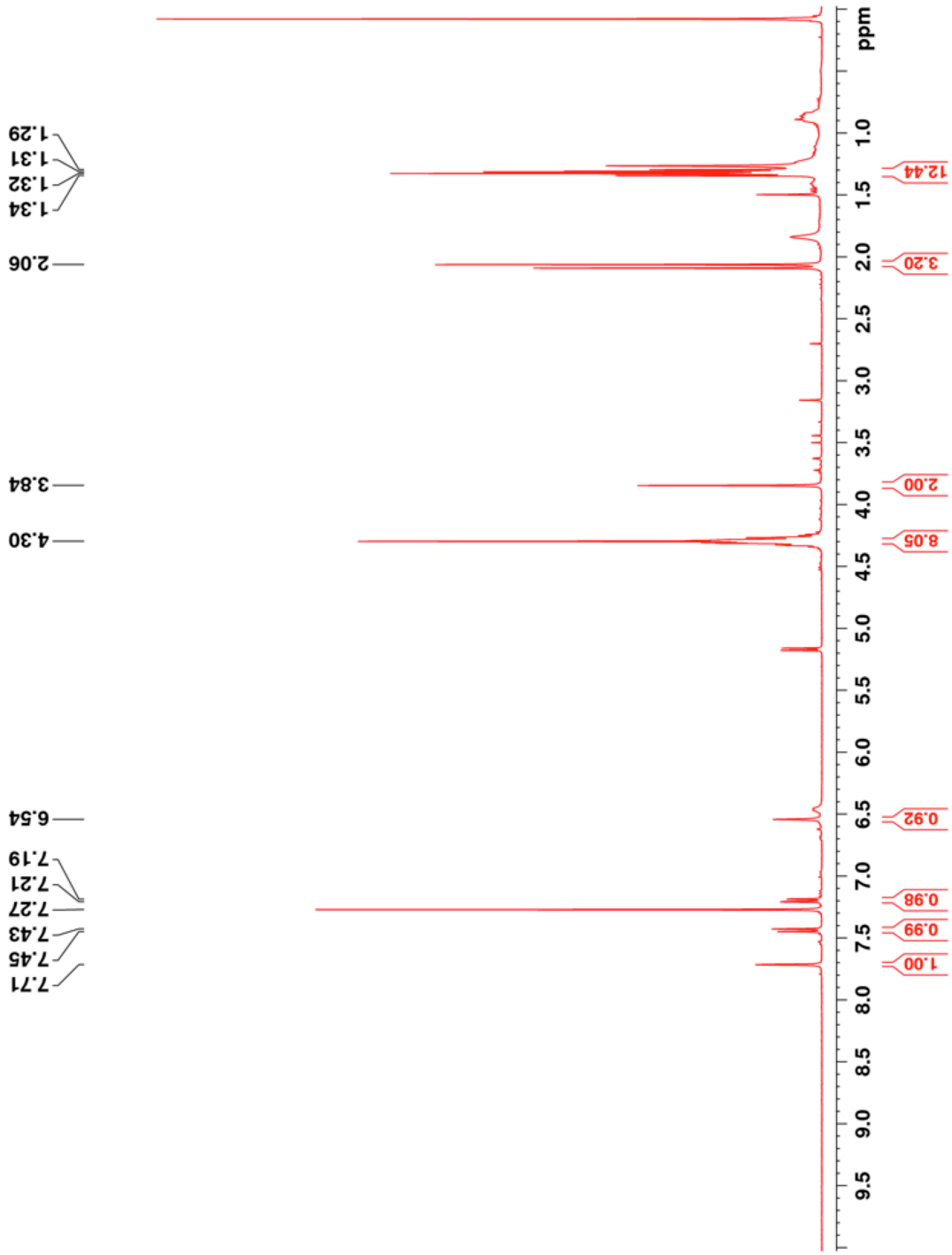


C^{13} NMR Spectra of compound **6**

H[1] NMR Spectra of compound **9**





H^1 NMR Spectra of compound **10**